DISPERSE, SURVIVE, PROPAGATE: MICROBIAL RESILIENCE IN THE SPOILAGE OF COMMERCIALLY PROCESSED FOODS

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DISPERSE, SURVIVE, PROPAGATE: MICROBIAL RESILIENCE IN THE SPOILAGE OF COMMERCIALLY PROCESSED FOODS

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Commercial spoilage in processed foods is an economic burden for manufacturers, and the etiological agents in these incidents represent some of the most resilient microorganisms in the food processing environment. Bacterial spores and the sexual spores of some filamentous fungi are the two most thermostable food-relevant propagules. Ascospores, hyphae, yeast cells, and conidia are each uniquely adept for dispersal in the production environment, while aciduric, thermophilic bacterial spore formers can survive and propagate under prototypical processing and storage conditions. Therefore, the goal of this project was to characterize the prevalence of microbial food spoilage incidents, identify the production conditions which select for particular spoilage organisms, and develop practices and controls to minimize the likelihood and severity of spoilage incidents. A survey of over 50 fruit and vegetable juice manufacturers revealed that 64% of respondents had faced quality threats from heat resistant molds and 78% were concerned with *Alicyclobacillus* spoilage. The vast majority (90%) of manufacturers indicated better control over microbial spoilage would increase profits and reduce waste. A prediction model was developed from an observational study assessing the ecology of fungi isolated from spoiled products

submitted by industry collaborators. The outcomes at terminal nodes following recursive partitioning were included if they represented a ≥15% probability, which resulted in a decision tree with actionable outcomes for industry. Two examples from this collection of observations were explored further. *Mucor circinelloides* spoilage of yogurt was characterized under different thermal treatments, incubation conditions, and preservative regimes. Depending on a company's control over sanitation programs and degree of risk aversion, maintenance of the cold chain or variable amounts of natamycin (>10 ppm) were determined to be sufficient intervention strategies to minimize spoilage from this post-processing, dimorphic contaminant. The spoilage potential variability of a collection of 53 food-sourced *Alicyclobacillus* isolates was also evaluated in various model systems. Citric acid was determined to significantly reduce the synthesis of guaiacol, the causative spoilage metabolite, which was produced variably across species from differing isolation sources. Collectively, these results may be useful in the development of in-plant quality controls and the selection of targets in validation and challenge studies.

BIOGRAPHICAL SKETCH

Abigail Snyder received her B.S. in Food Science and Nutrition from The Ohio State University in 2012. Her undergraduate thesis projects focused on nonthermal microbial inactivation strategies for application in fruit and vegetable products, and the development of analytical methods to detect adulteration in grape juice blends. This work was conducted in the labs of Professors Ahmed Yousef, Monica Giusti, and Luis Rodriguez. Following a summer appointment in the lab of Professor Randy Worobo, Snyder began her doctoral work in Food Microbiology with minors in Microbiology and Biochemistry. Her project has involved the integration of applied research with industry-facing extension to characterize and develop controls for microbial spoilage challenges in processed foods.

ACKNOWLEDGMENTS

Many thanks to my advisor, Randy Worobo, for his support throughout my graduate program in the areas of research, teaching, and extension, and to my committee members John Helmann and Joe Peters for their guidance as my minor advisors. Additionally, Martin Wiedmann, my field appointed committee member, and Olga Padilla-Zakour, my department chair and the director of the Cornell Food Venture Center, were also essential collaborators in this process. Other members of the food microbiology extension team, Kim Bukowski and Elizabeth Sullivan, facilitated interactions with our industry collaborators on my behalf. Finally, many thanks to the academic and research staff, notably John Churey, Sarah Lincoln, and Erin Atkins, in the Department of Food Science, for their constant and reliable help.

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CHAPTER 1

Nature abhors a vacuum: Highly diverse mechanisms enable spoilage fungi to disperse, survive, and propagate in commercially processed and preserved foods

Abstract

Food processing, packaging, and formulation strategies are often specifically designed to inhibit or control microbial growth to prevent spoilage. Some of the most restrictive strategies rely on pH reduction, preservatives, water activity limitation, control of oxygen tension, thermal processing, and hermetic packaging. In concert, these strategies are used to inactivate potential spoilage organisms or inhibit their growth. However, for the select microbes that can overcome these controls, the lack of competition from additional background microbiota helps facilitate their propagation. Fungi, as a group, represent some of the most resilient spoilage organisms capable of overcoming the control strategies utilized by the food industry. Various fungal propagules are rapidly dispersed by water and air, survive under extreme conditions, and sustainably increase in biomass. Some fungal species are known to persist under the most extreme physicochemical conditions and thermal processing regimes used in commercial food production. Yeast and filamentous fungi have proved particularly well suited to dispersion and cross-contamination within the food processing infrastructure. Due to these diverse structural features and survival mechanisms, fungal species are well adapted to particular ecological niches that enables them to contaminate and spoil commercially processed foods.

1.1 Fungal spoilage of processed foods

The burden of fungal food spoilage on the food industry should be evaluated using several different metrics. Fungal growth, though typically not a food safety concern, contributes to waste and loss throughout the supply chain, is the cause of recalls and perishability, and can lead to consumer dissatisfaction and mistrust in food manufacturers and retailers. Globally, approximately 25% of food is wasted or lost due to post-harvest microbial spoilage (Gram et al., 2002). There is a significant need for quantitative assessments of product-specific food waste due to various factors, and for reports of microbial spoilage issues. In comparison, food safety issues are reported to regulatory agencies and extensive data exist on the incidence of foodborne disease, its economic impact, and the prevalence of specific illnesses associated pathogenic organisms.

Recent and persistent examples of microbial food spoilage recalls and product withdrawals, in addition to emerging data on food waste that occurs as a result of spoilage, substantiates the need for continued efforts in the area of food mycology. Decisions surrounding quality control programs are made throughout the manufacture process, from determination of ingredient quality specifications prior to receipt and establishment of the processing conditions, to shipping and handling practices and sanitation programs. Manufacturers often face fungal spoilage issues on raw ingredients and in finished products (Table 1.1). The processing history, packaging, formulation, and composition of the food all impact the likelihood of microbial spoilage and the relevant organisms. It is often the case that the intervention strategies used to control foodborne pathogens contribute to the selection of food spoilage microorganisms

(Krebs et al., 1983). Many of the most resistant spoilage organisms, capable of growing in seemingly inhospitable environments, are fungi (Table 1.2).

Table 1.1: Common names for a selection of commercial food spoilage issues and the associated causative fungus and quality deviation

Commercial name	Description	Scientific Name	Ref.
Machinery mold	Accumulation of white or black mold on processing equipment. Can cause off-flavors and dextran production, has been associated with dairy, fruit and vegetable, and frozen food processing environments.	*Geotrichum candidum*	86
Chalk mold	Surface spoilage of bread identifiable by white or pink patches.	*Pichia burtonii*	73
Thread mold	Fungal growth in the small wrinkles of plastic packaging, often associated with cheese.	Mycelial growth of several common molds - *Phoma, Aspergillus* spp., *Penicillium* spp.	45, 81
Puffballs	Mold growth at the bottom of beverage containers. Sometimes identified as a "mouse in my product" by consumers in their customer complaints due to the dense, filamentous nature.	Mycelial growth, often associated with *Paecilomyces (Byssochlamys)* growth in heat treated juices	
Mat	Mold growth at the top of beverages or other fluids. Associated with beer, pickles, and other fermented vegetables.	Pellicle - Aerobic growth of one or more fungus	
Black mold	Powdery, black contamination resulting from profuse conidiation.	*Aspergillus niger, Stachybotrys*	1
Bloated	Swollen container resulting from gas production (often but not always CO_2) associated with fermentation and growth. In extreme cases the package will rupture under the increased pressure.	Yeast spoilage, very rarely filamentous fungi	5
Belly Burst	Anchovy belly explodes due to proteolysis and gas production.	Combination of mold, yeast, fungi	38
Film Yeast	Thin pellicle growth on the high oxygen interphase between product and headspace.	Several oxidative yeasts form films - *Candida, Pichia*	4
Food Splash	Spoilage of shelf-stable, hot filled products due to product splashing on the lip of the container, compromising the seal integrity and creating a conduit for spoilage fungi.	Typically aerobic, heat sensitive molds - *Aspergillus, Penicillium, Cladosporium*	10
Leaker Spoilage	Container bloating due to gas production of post-processing contaminants introduced during seam defects, pressure control, and cooling water sanitation.	Gas producing spoilage fungi and bacterial spore formers	10

The impact of fungal food spoilage in the processed food category is significant, given the inherent selection criteria imposed on the environment by formulation and processing. In fact, fungi have been shown to cause spoilage in products which were not believed to support growth (Gunde-Cimerman et al., 2009; Turk et al., 2007; Gostincar et al., 2009; Cavicchioli et al., 2002). Fungi proliferate in foods with extremely limited water availability, and conversely, they have been shown to cause spoilage in foods that are very nearly pure water. Previous reports have shown that mineral water and aqueous solutions of organic acids can support fungal growth, despite the seeming lack of all but residual nitrogen (Cabral and Pinto, 2002; Criado et al., 2005). Similarly, fungi have been shown to survive and ingress into what are considered commercially sterile foods due to the extreme heat resistance of some propagules and the profuse aerosolization of others. Although food spoilage is the primary concern with fungi, several issues related to safety exist, yet are not often discussed. Fungi are not considered biological hazards, but several otherwise commensalistic fungi are opportunistic pathogens (Benedict et al., 2016). Additionally, acidified products, by definition, contain organic acids which may support the growth of fungi (Piper et al., 2002). The breakdown of these acids by fungal metabolism increases the pH of the product, and can facilitate the growth of pathogenic bacteria (Black and Barach, 2015). Mycotoxins are considered chemical hazards, but are characterized primarily by their formation in raw agricultural products, the ingredients in commercial food production, rather than as hazards commonly associated with finished product spoilage (Binder et al., 2007).

Imposition of various processing and formulation strategies reduces the diversity

of microorganisms capable of proliferation under those conditions. The parameters surrounding food production strategies are frequently designed to control bacterial pathogens, but many spoilage organisms are more resilient. Perhaps the earliest example of a commercial processing technique designed to control spoilage microorganisms is the water bath canning method pioneered by Nicolas Appert in the late 1700's for use by the French military to preserve food for long expeditions (Black and Barach, 2015). The challenges posed by fungal food spoilage today are still augmented by the demands of emerging food production and distribution systems, although our ability to identify and control specific spoilage organisms has improved since Appert's time due to the advent of molecular techniques and international taxonomic consensus (Filtenborg et al., 1996). Much of the scientific literature in food mycology has been produced by a small group of dedicated researchers. Beuchat and Splittstoesser, U.S. based researchers, published extensively in the 1970's and 1980's on the processing conditions and physicochemical properties which supported survival and propagation of spoilage fungi. Pitt and Hocking are prominent Australian mycologists who produced a great deal of the founding literature regarding xerotolerant fungi. Samson and his collaborators Frisvad, Houbraken, Hoekstra, and Dijksterhuis, based largely out of the Westerdijk Fungal Biodiversity Institute in the Netherlands, have published some of the seminal work on fungal taxonomy and structure-function. Much of the discussion in this review is based on the findings of these researchers and their collaborators. As many decades worth of literature has shown, the diverse mechanisms possessed by fungi to disperse, survive, and propagate in food systems continue to make them some of the most troublesome spoilage organisms faced by food

processors.

1.2 Fungal structural physiology supports survival and dispersal

The diversity of reproduction and growth methods available across the fungal kingdom contributes to the dispersion and survival of the cells in harsh and changing environments. Vegetative growth can occur in either a filamentous or yeast phase, often depending on environmental conditions, and the differences in the cellular structure between these forms can variably increase their transmission through water, air, or a host vascular system. Generally, fungi can reproduce clonally through asexual reproduction and recombinantly through sexual reproduction (Fig. 1.1). Although variations in process and naming conventions exist across the fungal kingdom, those for Ascomycota specifically relevant to the food industry are discussed here.

Asexual reproduction, the reproduction strategy used by bacteria and other prokaryotes, generates spores that are genetically identical to the parent. These asexual spores include conidia, blastospores, and chlamydospores, depending on how they are formed. Sexual reproduction, in contrast, yields ascospores that can be highly resistant to environmental stresses and genetically distinct from the two parental mating types involved in their production. Asexual reproduction occurs more frequently and is typically a part of a general growth and dispersion strategy, whereas sexual reproduction is often induced only under conditions of increasing stress.

Sex occurs widely in the fungal kingdom, although only an asexual reproduction strategy has been observed for some species of foodborne fungi (Wyatt et al., 2013). A sexual cycle was only just described in 2009 for *Aspergillus fumigatus,* despite the extensive work that had previously been done on this human pathogen (O'Gorman).

Sexual cycles for fungi may elude identification if the environmental factors which induce their initiation are difficult to replicate or unlikely to be introduced in the laboratory. *Candida,* for example, was thought to only replicate asexually until a sexual cycle was discovered in cells in the mammalian host (Hull et al., 2000). Additionally, sex requires compatible mating partners and may only occur among a confined substrata of species. The low abundance of compatible partners is an indication on an ecological level that a species is experiencing a slow decline in fertility, which may be accompanied by loss of the genetic elements responsible for sexual reproduction (O'Gorman). Some putatively infertile strains of the Heat Resistant Mold (HRM) *Paecilomyces* (*Byssochlamys*) have been identified (Houbraken et al., 2008). A schematic summarizing the interconversion among these cycles is presented in Fig. 1.1, and the costs and benefits to spoilage potential of the different strategies and cell types are discussed below.

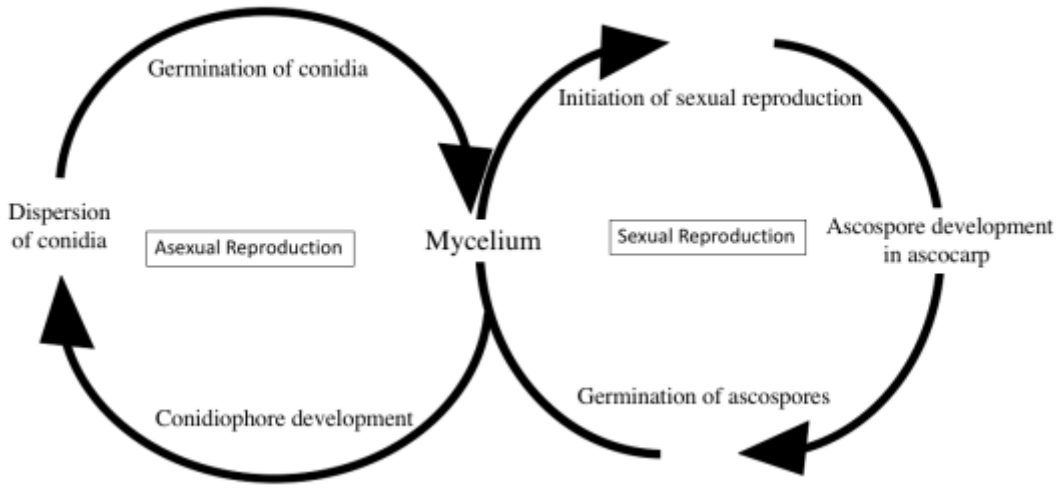

Figure 1.1: Generalized reproduction schemes for filamentous fungi in Ascomycota

Ascospores

Ascospores are the product of sexual mating between compatible partners, and are the most resilient of all the fungal cellular forms - they are the propagule associated with spoilage of canned foods by HRM. Indeed, sexual cycle induction is often initiated in response to environmental stresses and yields highly resistant cells capable of persisting under hostile environments until conditions are more favorable for growth, at which time they will germinate a more genetically diverse population potentially better equipped to withstand changing conditions (Goddard et al., 2005; Conner and Beauchat, 1987a). Sexual reproduction increases genetic diversity through mating and recombination, although some fungi are capable of self-fertilization. Mating compatibility generally refers to two fungal entities which possess the complimentary genetic elements needed to facilitate mating, cytoplasmic fusion of the vegetative cells followed by nuclear fusion and meiosis (Nieuwenhuis et al., 2013). Fungi can be either heterothallic or homothallic - i.e. the fungal entities that contain compatible mating elements are either two separate individuals (heterothallic), as in the animal kingdom, or found within a single individual at two different genetic loci (homothallic) (Kurtzman et al., 1980). Heterothallic fungi more readily incorporate genetic diversity into the population since two unique individuals are required for sexual reproduction, but the frequency of finding a compatible mating partner may be lower than for homothallic fungi in which a single individual possess all the genetic elements necessary to produce ascospores (Goddard et al., 2005). Homothallism still involves recombination, an opportunity to bleed off deleterious mutations from the population (Bruggeman et al.,

2003). The majority of fungi within the family Trichocomaceae are homothallic, although there are many exceptions and variability exists within genera (Wyatt et al., 2013). For example, *Paecilomyces variotti* (*Byssochlamys*) is a heterothallic species of HRM, closely related to *Paecilomyces niveus* (*Byssochlamys*) which is homothallic (Houbraken et al., 2008). Idiomorphs are the bipolar alleles of the mating type locus (MAT 1/MAT 2, MATa/MATα, etc.) encoding transcription factors regulating sexual reproduction via pheromone and pheromone receptor expression in Ascomycota (Gomes-Rezende et al., 2012). One mating type capable of secreting a pheromone is recognized by the receptor of a compatible mating partner, which induces the partners to approach one another and form sexual structures to facilitate plasmogamy. This system varies among more diverse members of the fungal kingdom. Many Basidiomycota, for example, utilize a tetrapolar mating system to increase the number of potential compatible partners (Bakkeren and Kronstad, 1994). Mucormycotina, although bipolar, develop mating structures in response to the pheromone trisporic acid. Compatible mating types (SexM/SexP) synthesize different and incomplete precursor elements such that coordinated synthesis of trisporic acid itself indicates the presence of elements expressed from both compatible partners (Sutter et al., 1973).

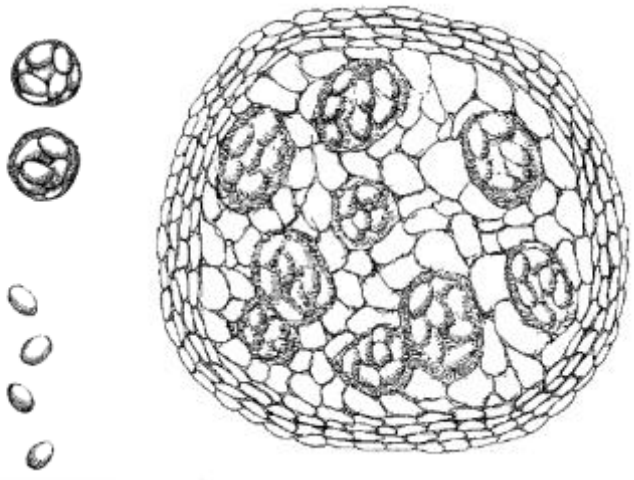

Figure 1.2: Generalized sexual spore structure for Ascomycota. Loose ascospores (spores) are typically packaged as octads in a sac-like ascus that contains the product of four meiotic and a single mitotic division. The asci are contained by tightly woven hyphae in a larger fruiting body, the ascocarp (or ascomata or sporocarp), as shown in the cleistothecium (globose ascocarp) above.

Sexual spores, or ascospores, develop inside various external structures during maturation (Fig 1.2). They are typically packaged in units of eight in a sac-like ascus, which may provide an additional barrier of protection from the environment. Ascospores themselves have a thick cell wall, > 0.5 μm, sometimes ornamated with proteinacous spikes, which serves as a protective barrier (Dijksterhuis, 2007). The shape of ascospores varies by species, but many food-relevant fungi are round to ellipsoidal and 8-14 μm (Wyatt et al., 2013; Dijksterhuis, 2007). The spores in a single ascus are a tetrad, four pairs of twins (Codon et al., 1995). Although the exact contribution of the ascus to heat resistance is not clear, ascospores are often liberated before use in experiments through sonication or shear force (Michener and King, 1974). Spore age and growth conditions also influence the formation and heat resistance of ascospores. The variability in ascospore production has indicated that regulatory control over sexual reproduction is attuned to various environmental conditions and is strain specific

(Conner and Beauchat, 1987c). The exact age of developing ascospores that corresponds with optimal stress tolerance is also strain specific. Morphological shifts that correlate with this aging process include changes to the inner cell wall and development of the lateral ridge that bifurcates the exterior of some ascospores (Conner and Beauchat, 1987b). Asci are typically enclosed in larger structures, generally called fruiting bodies. Fruiting bodies in Basidiomycota include mushrooms, and are structures in which groups of asci are nested in dense hyphae during development. In Ascomycota, the fruiting body is variably called the ascocarp, sporocarp, or ascomata and it is delimited from the surrounding hyphae by a peridium, or outer skin (Sohn and Yoon, 2002). Moreover, the ascocarp can be described by its shape. Common forms include cleistothecial (round or globose, spores are released through its degradation), perithecial (flask-shaped, spore are released through the neck), and apothecial (cup-shaped, spores are released from the exposed surface) (Moore-Landecker, 1992). Most of the highly thermotolerant ascospores are formed in cleisothecial ascocarps (Wyatt et al., 2013; Wyatt et al., 2015a). Ascospore containment in cleisothecia increased the thermoresistance of *Talaromyces flavus* to 30 min at 100°C, while loose ascospores were inactivated more readily at <95°C (van der Spuy et al., 1975; Katan, 1985). Cleistothecia are embedded in a loose mycelial network (Conner and Beauchat, 1987b). The fruiting body of *Paecilomyces* (*Byssochlamys*) is notable for its loose, unstructured formation, naked asci are clustered throughout the mycelial network (Brown and Smith, 1957). Ascospore morphologies, and their surrounding structural features, have been crucially used in characterization and identification of species. Prior to molecular typing, isolates were classified based on these structural features which lead to a

redundant naming system as sexual (teleomorphic) and asexual (anamorphic) cells of the same organism were classified independently of one another. Today, mycologists are moving towards a "one fungus, one name" holomorphic convention wherein assignments are based on several factors regarding the potential synonymic names. However, some names that are being eschewed still carry phenotypic implications that are recognized by the food industry. Therefore, synonyms for some of these isolates (e.g. *Paecilomyces/Byssochlamys*) are used in this text.

Conidia

During vegetative growth in filamentous fungi, a subset of mycelial filaments will form specialized, aerial structures called sporangium, or condiophores, which bear chains of asexual spores, referred to as conidia among Ascomycota. The formation of sexual vs asexual spores is governed by Kleb's Law which states that the ratio of sexual to asexual spores formed increases as conditions becoming increasingly growth limiting (Dahlberg and Van Etten, 1982; Conner and Beauchat, 1987c; Codon et al., 1995). Although conidia are not as resilient as most ascospores, they tend to be more resistant to environmental stresses than vegetative hyphae, often possessing a thicker cell wall to prevent lysis during phase transitions between wet and dry environments during dispersion (Dijksterhuis, 2007; Wyatt et al., 2015b). And, like ascospores, conidia can remain dormant until conditions become more hospitable. Conidia are formed for reproduction, but other asexual structures, like chlamydospores, are formed for survival and are discussed below. Conidia are particularly relevant to the food industry as the most common contaminating propagule within the production environment (Pitt and Hocking, 2009).

The condiophore bearing the conidia develops from an erect branch of the parental thallus and may project up to 1 mm above the substrate surface (Trinci and Banbury, 1967). The conidiophore variably branches into one or more rami that themselves may be branched into one or more metulae which may bear one or more phialides (or sterigmata). The complex branching pattern of the conidiophore has been used in species identification, particularly among Penicillia and Aspergilli (Pitt, 1974). Fig. 1.3 shows clusters of three phialides borne on a single branch of the conidiophore. Phialides are the flask-shaped penultimate projections of the asexual reproduction structure from which chains of conidia are pushed through the neck of the phialide, the oldest conidium at the end of the chain.

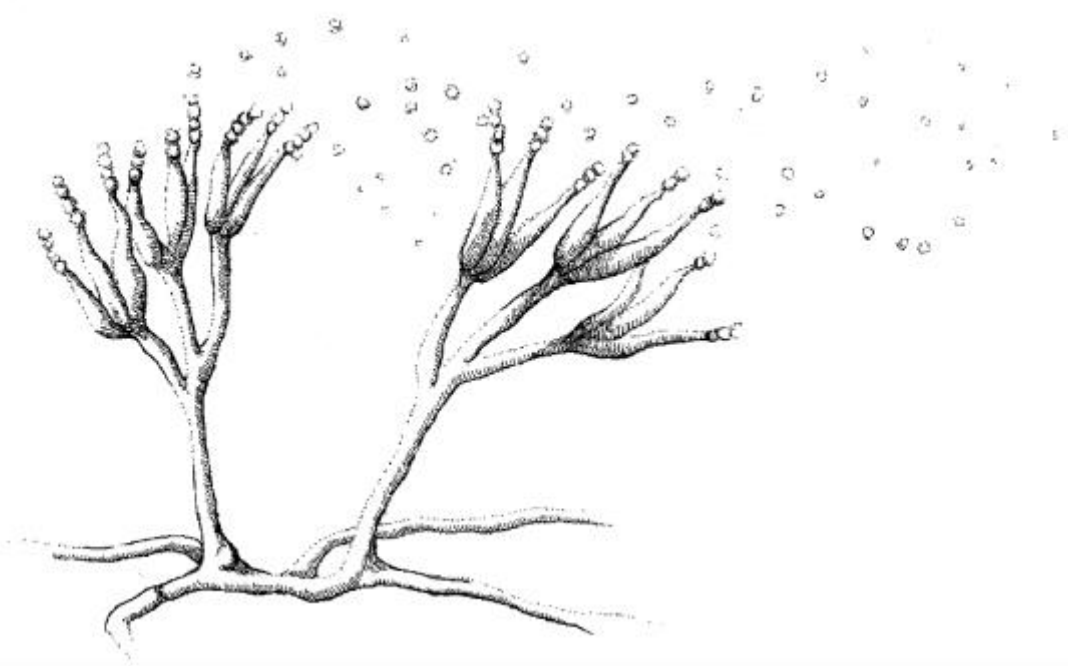

Figure 1.3: Generalized representation of asexual spore formation and release in some species of Ascomycota. Above, conidia form in long chains at the end of brush-like specialized hyphae called conidiophores which vary in their branching complexity. The swollen flask-like structures which bear the conidia are known as phialides. This profuse conidiation is emblematic of the asexual reproduction strategy utilized among many members of the Trichocomaceae family, common food spoilage fungi, to increase dispersal.

The chains of conidia borne on aerial conidiophores extend above the laminar airflow near the surface of the growth substrate (Wyatt et al., 2013; Van Leeuwen et al., 2009). Co-ejection likewise propels spores higher into turbulent air streams due to drafting effects than would asynchronous release (Roper et al., 2010). Some fungi may produce attractants and rely on insects or other intermediates for dispersal. Wynns (2016) speculates that spore structures co-evolved in bee habitats to promote insect-mediated transmission. Conidia are much smaller than ascospores, only about 2-3 μm in diameter, and are similarly lightweight and contain low moisture, which facilitates their dispersion by air streams with only slight agitation (Kwon-Chung and Sugui, 2013). The conidia of some fungi are evolved to support dispersion primarily through water vectors, as opposed to air. The conidiophores of waterborne-conidia are diminutized and their spores are often less resistant to desiccation and heat inactivation. (Van Leeuwn et al., 2009). *Cladosporium* is the most prevalent airborne fungus, due at least in part to the structure of its conidia (Crous, 2007). *Cladosporium* is often a contaminant in the processing environment and its spores are small, dry, and heavily pigmented, protecting them from ultraviolet (UV) inactivation (Pitt and Hocking, 2009). The conidia of many common foodborne fungi are pigmented to protect against UV. In fact, the *Penicillium* and *Botrytis* fruit and vegetable pathogens are often referred to in industry as “blue/green mold” and “gray mold” due to conidia pigmentation (Conner and Beauchat, 1987b). Pigmentation is considered a virulence factor among some plant pathogens, and plant infection with powdery and downy molds is increasingly problematic in UV blocking greenhouses (Jahn et al., 1997). As a consequence, conidia which express high levels of melanin are better able to survive during extended

exposure to light (Tiedt, 1993). The hydrophobic nature of many conidia also facilitates dispersion by air and water and is due to hydrophobins which decorate the surface of conidia (Kwon-Chung and Sugui, 2013). Hydrophobin rodlets are produced during spore egress from the phialide and adhere to the melanin containing layer on the nascent spore (Beever and Dempsey, 1978).

Hyphae

Fungal vegetative growth occurs through filament extension, known as apical growth. Hyphae are extended outward from the tip by the Spitzenkörper (literally, "tip bulb" or "apical body"), a vesicle complex which assembles intracellularly near growing hyphal tips to deliver the building blocks for cell wall biosynthesis (Reyanaga-Pena et al., 1997). Vesicles are transported along the actin cytoskeleton which polymerize to provide targeted delivery to the nascent cell membrane at the hyphal apex (Riquelem, 2013; Berepiki et al., 2011). In general terms, the hyphal cell wall is composed of chitan, glucan, and protein and extensive cross-linking occurs among these three components. Chitan is arranged in an ordered layer around the cell membrane, and on top of the chitan is a layer of β-glucans. Glycoproteins extend throughout the cell wall, some are tethered to the cell membrane itself. The outer cell wall may be composed of mannan (Gow et al., 2012; Levitz 2010).

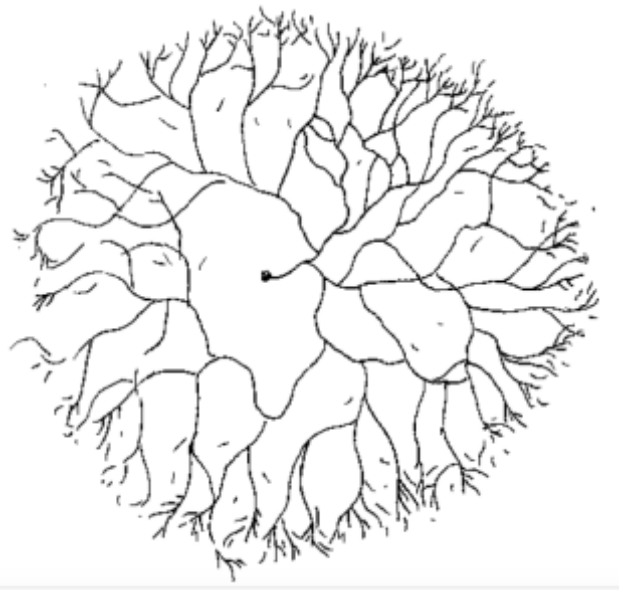

Figure 1.4: A mature mycelium is composed of an extensive network of interconnected hyphae. Hyphae are thin, filament-like vegetative fungal growth structures through which nuclei, other organelles, and nutrients can travel during growth and homeostasis. Growth is apical (occurring from the tip of the hypha) and cross connections among neighboring hyphae only occur later in mycelial development. A single, connected collection of hyphae is considered clonal and is variably referred to as a colony, mycelium, or thallus.

Hyphae of foodborne spoilage fungi are often septate, meaning the filaments are segmented by cross-walls (Shen et al., 2014). In contrast, the hyphae of Mucormycota are often coencytic, lacking septation. Septa contain central pores which allow for the sharing of cytoplasmic contents including nuclei and nutrients, which aids the extending hyphal filaments in colonization of nutrient pore environments (Wyatt et al., 2014). Under conditions of stress, cell wall damage, or if the fungus senses invasive nuclei, the pore can be closed by the placement of a membrane bound Woronin body across the pore (Markham and Collinge, 1987). Basidomycota possess a similar structure called the septal pore cap (Driel et al., 2009). Branching and cross-connections among neighboring hyphae serve similar purposes and are also closely regulated by the cell. When the mycelium is young, hyphae extend outward without branches or cross-connections; however, older filaments can increase their fitness and ability to colonize

new and increasingly hostile environments by leveraging the resources of a network of hyphae. Cross-connections can be formed if two hyphae possess "vegetative compatibility." As with mating compatibility described above, vegetative compatibility is predicated on allelic interactions (Bayman and Cotty, 1991). However, with sexual reproduction, compatible mating types identify one another by genetic differences at sex-specific loci. In order for vegetative fusion to occur, the hyphae must possess the same allele, or the filament will seal its septal pore and initiate programmed cell death against the invading filament. Due to these extensive structural features, mature mycelia of Ascomycota are composed of a floccose mat of branched, cross-connected, and intertwined filaments through which nuclei stream freely. This has long provided challenges for food mycologists in quantification of cell growth. For single celled bacteria and yeast, plate counts of colony forming units are used effectively under the assumption that one colony approximately correlates to one cell. However, the notion of a single individual is harder to apply in filamentous fungi and may not be relevant to quantification of spoilage or growth. Plate counts have traditionally been used within the field, with the associated caveats. Plate counts of filamentous fungi are often correlated with the type and degree of physical disruption, such as stomaching, during sample processing. Quantitative PCR using a single copy amplicon as the target may more accurately quantify the number of nuclei, but identification of that target may be specific to the individual and not representative of spoilage. Quantification of mycelial biomass may be the most reflective of spoilage, but extraction from complex food matrices can be difficult and comparison across growth substrates may be limited as hyphal density is correlated with the physicochemical properties of the matrix, notably

water availability.

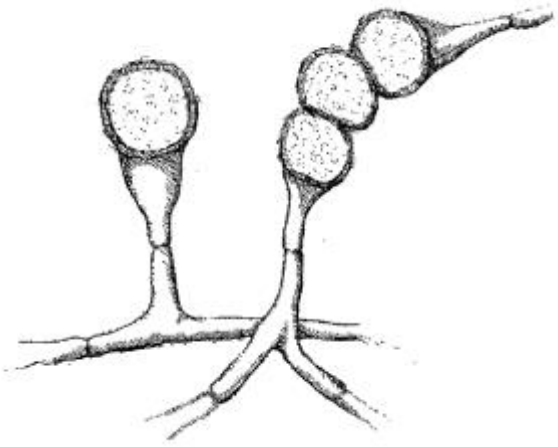

Figure 1.5: Many Ascomycota species produce thick-walled resting spores that have increased thermal resistance and desiccation tolerance. Chlamydospores are formed endogenously by vegetative hyphae terminally or intercalary, within the filament. They are often referred to as asexual "resting spores," since they function for survival instead of dispersal, as in the case of conidia.

As described above, hypha can transform into a specialized structure known as a conidiophore or sporangium for replication. Hyphae can additionally undergo changes to produce asexual spores which are for survival and generally referred to as "resting spores." Chlamydospores (Fig. 1.5) can be formed internally or on the terminus of a hypha when the cell wall closes off the septal units, nutrient storage compounds are accumulated in the cytoplasm, and the cell envelope forms a thickened barrier against environmental stresses. Chlamydospores have been observed in *Paecilomyces/Byssochlamys, Mucor, Candida,* and *Fusarium* (Elad and Baker, 1985). Sclerotia are masses of hyphae with thickened cell walls. The sclerotia of *Penicillium* have been isolated from spoiled canned blue berries having survived at least 4.5 min at 85°C (Tournas, 1994). Although resting spores may have increased resistance to food processing strategies, they are formed at a low frequency in developing mycelia.

Ascospores remain the most frequently encountered heat resistant propagule within the food industry (Tournas, 1994).

Yeast

Many fungi possess a unicellular or yeast growth phase. Within food microbiology texts, fungi are often differentiated as either yeast or molds, although these are cell structure not taxonomic distinctions. However, many of the yeast of concern within the food industry (*Saccharomyces, Pichia, Candida, Rhodatorula, Torulaspora, Zygosaccharomyces, Brettanomyces*, etc.) are classified within the family Saccharomycetaceae. These fungi grow and replicate as planktonic yeast under conditions normally encountered within food production. *Candida,* for example, is a spoilage yeast commonly isolated from human epidermis and built environments, and it has been associated with the spoilage of yogurt, bread dough, and sugary beverages. *Candida albicans* lives commensally on human skin, but is an opportunistic human pathogen. During infection, *C. albicans* hyphae penetrate host cells (Gow et al., 2012). Hyphal growth is induced under temperatures approaching 37°C in sera containing medium. After local colonization of the host cells, *C. albicans* yeast are transmitted through the host vascular system in a systemic infection. Environmental ques also trigger changes between the dimorphic growth phases of *Mucor circinelloides* as was reported in a national recall of Greek yogurt. *M. circinelloides* yeast-like cells caused container bloating due to CO_2 production in sealed yogurt containers, but consumers who opened containers and returned them to refrigeration noticed mycelial growth in their product (Snyder et al., 2016). *M. circinelloides* grows as a yeast under oxygen and hexose limiting conditions, and as hypha under ambient conditions. Most spoilage

fungi, though, grow and are recognized as either yeast or hyphae under all food spoilage relevant conditions. The unicellular growth phase for yeast introduces several key factors into their transmission, survival, and propagation within food systems.

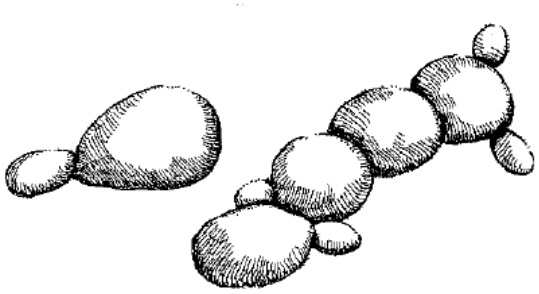

Figure 1.6: Generalized representation of yeast cells reproducing by budding. Yeast are single-celled planktonic structures that reproduce through either budding or fission. Colony and cell morphology appear similar to bacteria, except yeast are usually larger (~10 μm diameter). Above, blebs (small buds) form as part of an asexual reproduction cycle that will eventually result in clonal blastospores.

Yeast are not likely to be introduced into a product by air, but are instead likely to contaminate products via aerosolizing in a water or food vector (Pitt and Hocking, 2009). For this reason, sanitation within the production environment to minimize condensation, standing water, splash, and regions within processing equipment with little agitation (i.e. dead legs) is essential in preventing yeast spoilage (Lawlor et al., 2009). Yeast are often associated with spoilage of beverages and other fluid products since unicellular microbes can better disperse in fluids, and because yeast spoilage occurs more readily in low oxygen environments like the subsurface anaerobic conditions in fluids (Pitt and Hocking, 2009). No food-relevant yeast is considered heat resistant, Splittstoesser (1996) stated that most yeast are inactivated within 1 min at 55°C. Ascospores of yeast are indeed more heat resistant than vegetative yeast, but are still inactivated under most processing conditions (Splittsoesser, 1996; Conner and

Beuchat, 1987a). Asexual yeast reproduction can occur through budding or fission. The majority of food spoilage yeast utilize budding in which blastospores or blebs form from an asymmetric division in a parental yeast. As the nucleus of the parent yeast undergoes meiosis and the daughter nucleus migrates into the blastospore, the cell wall closes around the new cell leaving a bud scar on the parent (Li and Murray, 1991). This process repeats again as both mother and daughter yeast form buds, a process that can occur relatively quickly under optimal conditions.

Table 1.2: Resistance to physicochemical properties of food for select fungal species

Species	Conditions Supporting Growth	Food Spoilage Implications	Ref.
(Paecilomyces) *Byssochlamys spectabilis* *B. fulva* *B. nivea*	 O_2 = 0.5% O_2 = 0.27% O_2 = 0.5%	Survival of hot fill processing leads to spoilage in reduced oxygen headspace during shelf-life.	33
Cladosporium cladosporiodes	A_w=0.85	Psychotropic growth on cured meats and cheeses. Ubiquitous.	53
Xeromyces bisporus	A_w=0.65	Confections, dried fruits, baked goods, preserves, etc.	63
(Aspergillus) *Eurotium herbariorum* *E. repens* *E. amstelodami*	 A_w=0.85 A_w=0.70 A_w=0.71	Optimal growth on low and intermediate water activity foods, dried goods and preserves. *Aspergillus* spp. are ubiquitous.	44
Penicillium chrysogenum *P. roqueforti*	Lactic, acetic, sorbic acid metabolism A_w=0.82	Fermented dairy products, use of these preservatives selects for these spoilage fungi. Ubiquitous.	6, 37
Zygosaccharomyces baillii	A_w= 0.85 Sorbic and benzoic acid metabolism	High sugar content products, syrups and juice concentrates. Preservative use selection.	54
Candida magnoliae	pH=1.75	High acid beverages and food.	10

1.3 Inactivation and survival under conventional and novel food processing systems

The most common microbial inactivation strategy used in the food industry is thermal processing. Although the propagules discussed above vary significantly in terms of their heat tolerance, the thermal tolerance of some fungal cells remains a significant challenge for food processors. The ascospores of *Paecilomyces (Byssochlamys), Aspergillus (Neosartorya),* and *Talaromyces* are the most resilient eukaryotic cells encountered by food processors. Most notably, these ascospores are known for their extreme heat resistance and are the cell types which gives rise to the functional category HRM. The ecological basis for this extreme stress tolerance is poorly understood, but it has been theorized that heat resistance allows for ascospore survival during forest fires (Fravel and Adams, 1986). Indeed, fungi are some of the first organisms to recolonize the decimated forest and activation of the spore germination process often requires initiation by some external heat shock. The extreme heat resistance of ascospores is associated with the thickness of the cell wall and the density of the cytoplasm. The biophysical attributes associated with abiotic stresses are well characterized in the recent work of Wyatt et al. (2013, 2014, 2015a, and 2015b) in which several central mechanisms for thermal tolerance are described.

The structural features of ascospores are described in section two, but in addition to the impervious cell wall itself, accumulation of compatible solutes in the cytoplasm may also help stabilize the cell envelope during dehydration by helping to preserve protein conformation and prevent membrane leakage. “Compatible solutes” are those small molecules which do not impede cellular function if accumulated at high

levels intracellularly, and it has been demonstrated that this strategy provides protection against thermal destruction, freezing, and desiccation (Wiemken, 1990). In fact, similar strategies are employed by plants for survival during freeze-thaw cycles. The non-reducing disaccharide, trehalose, and the sugar alcohol, mannitol, are two compatible solutes most relevant to stress tolerance in HRM (Dijksterhuis and de Vries, 2006). Trehalose has previously been identified in model yeast systems for its role in stress tolerance where its synthesis is upregulated under heat shock (Singer and Lindquist, 1998). Trehalose, or related molecules with a trehalose core, are the dominant solute sugar that accumulates during stress in vegetative cells (Managbanag and Torzilli, 2001; Wyatt et al., 2015a) and during ascospore development (Conner and Beuchat, 1987a).

Cytoplasmic density, as a general feature, is the summation of several biophysical phenomenon contributing to ascospore heat resistance. Ascospores have decreased water content compared to vegetative hyphae, and coupled with the presence of accumulated solutes, the cellular cytoplasm in sexual spores can be highly viscous (Dijksterhuis et al., 2007). Cytoplasmic viscosity has been measured in several species and varies by growth conditions (Connor and Beuchat, 1987c). The presence of the concentrated solute serves as a scavenger for reactive oxygen species (ROS) that form during dehydration and stabilize intercellular structures (Shen et al., 1997; Wyatt et al., 2013). Guiding principles for this phenomenon are the water replacement theory and glass formation within the concentrated cytoplasm. As water is lost during ascospore development, the network of hydrogen bonds for aqueous proteins is also lost and can lead to protein denaturation and cell death (Gervais et al., 1988). However, if those interactions can be substituted by the hydroxyl groups on sugars and sugar alcohols,

then protein structure will be stabilized and preserved. Similarly, the concentration of trehalose, specifically, leads to glass formation instead of crystallization inside the cell as water is removed (Sun and Leopold, 1997). As trehalose comes out of solution, the solid is amorphous instead of regular, and the viscosity of this gel slows diffusion rates and chemical reactions, like denaturation, ROS accumulation, and protein aggregation (Wyatt et al., 2013). The sugar alcohol mannitol is expressed at much lower levels in hyphae compared to ascospores (Wyatt et al., 2015b). Knock out mutants for mannitol expression did not impede vegetative growth, or formation of ascoscarps and asci for that matter, but ascospore development was limited if not totally aborted. Moreover, once ascospores begin the process of germination, sugar and sugar alcohols present in the cytoplasm at high levels are catabolized to support growth and heat resistance is lost concomitantly (Wyatt et al., 2015b). Thermal tolerance in developing ascospores is acquired after around two weeks growth in laboratory cultures, at which time cytoplasmic viscosity increases as compatible solutes are accumulated and bulk water is removed. Electron spin resonance spectroscopy using a non-interacting spin-probe indicated that the internal spore composition continues to change for several additional weeks as mannitol levels decreased relative to intracellular trehalose in *Aspergillus* (*Neosartorya*) *fischeri* (Wyatt et al., 2015b).

Critical limits and limitations for thermal processing

Commercial spoilage of canned foods by HRM has been documented in the literature for decades (Dijksterhuis, 2007). The genus for *Paecilomyces* (*Byssochlamys*), a commonly isolated HRM, was established at the start of the 20th century by an isolate taken from an alcoholic beverage. A second species was added to the genus in 1933 and

was described as “creating a nuisance in the fruit canning industry” (Brown and Smith, 1955). Thermal microbial inactivation processes are developed through validation studies which quantify the accumulated lethality for a target microorganism, typically a bacterial pathogen, by modeling the first-order relationship between microbial survivors and time at a given processing temperature. While this allows manufacturers to accurately estimate the efficacy of their process against their target, other potentially contaminating microbes not considered during validation may have different inactivation kinetics and persist. Conceptually this is obvious when refrigerated, pasteurized products spoil at the end of shelf-life. Although products were treated with a kill-step sufficient to reduce the pathogen of concern by 5 to 7 log, spoilage organisms which survived this treatment eventually proliferate and render the product unacceptable due to the byproducts of their metabolism. Fig. 1.7 illustrates the most likely fungal cell type (e.g. ascospore, conidia, hyphae, yeast) to cause spoilage under various processing conditions. Pasteurization processes often range from 63 to 70°C for several seconds for up to 30 min. Shelf stable products can be treated by hot fill, for acidic products, which usually involves treatment at 80-90°C for several minutes. Aseptic and retort processes involve heating above the boiling point of water. Predictive mycology is complex, determination of the most likely cell type is based on several factors, including prevalence, optimum growth, thermal tolerance, and likelihood of contamination. For example, fill temperature, storage temperature, and headspace volume can influence total package oxygen which likewise influences the probable spoilage organism. Subsequently, careful evaluation of production considerations is needed to determine the most relevant spoilage organism, and the potential to introduce

unintended issues when changing the process.

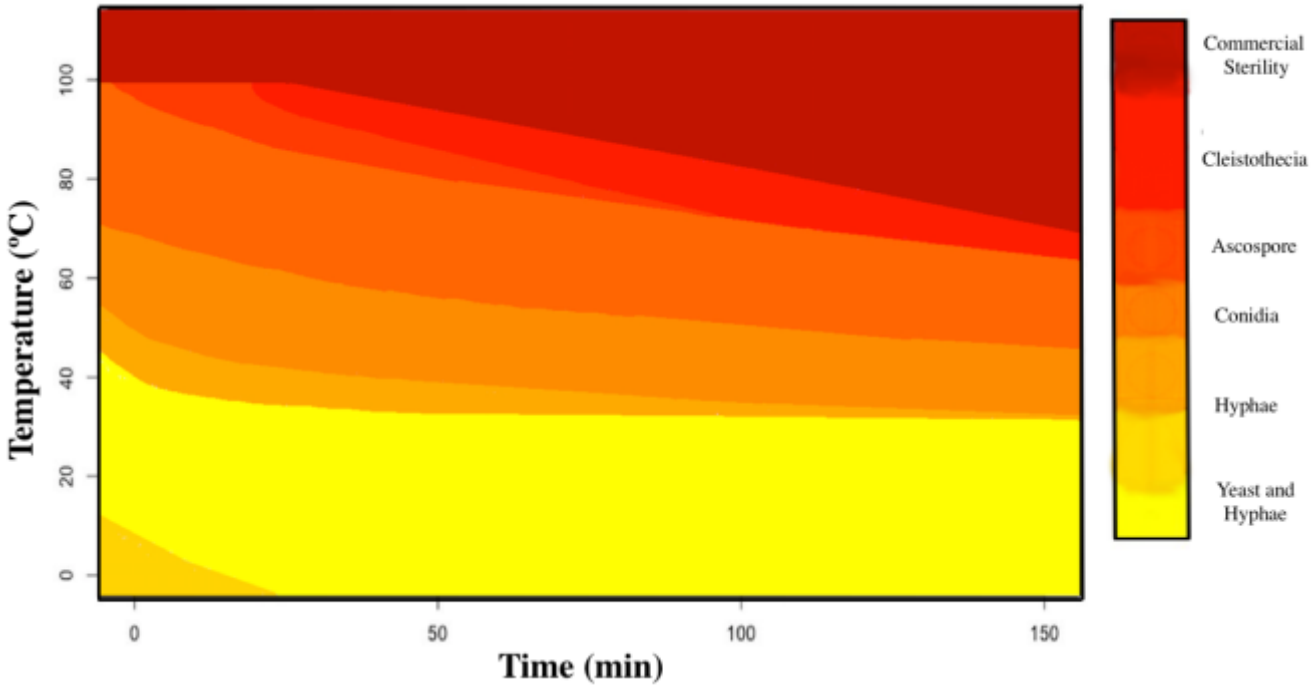

Figure 1.7: Fungal propagule survivors under increasing time and temperature treatments.

Cleistothecia enclosed ascospores of *Talaromyces* have survived up to 30 min at 100°C. However, commercial spoilage incidents have been more commonly associated with ascospore survival of processing conditions ranging from 70 to 90°C which can be tolerated for extended periods of time among several genera of HRM (Table 1.3). Under less intense thermal processes, more ubiquitous conidia are likely to contaminate products. *Talaromyces,* the most heat resistant genera of HRM, produces ascospores which can survive boiling water temperatures, and which are up to 17% trehalose by wet weight (Dijksterhuis et al., 2002; Wyatt et al., 2015a). By comparison, mannitol and trehalose account for around 5% by weight in conidia and mannitol is the predominant solute (Ruijter et al., 2003). Conidia, generally, are more heat resistant than vegetative

hyphae, so for products which undergo moderate heat treatment, yeast and vegetative hyphae may be eliminated while asexual spores survive, germinate, and cause spoilage. Among yeast, the most heat resistant ascospore formers are *Saccharomyces, Pichia,* and *Hanseniaspora (Kloeckera),* although none are considered spoilage threats in shelf-stable products (Pitt and Hocking, 2009). The principles governing thermal resistance in ascospores apply to other cell types as well, conidia possess thicker cell walls and denser cytoplasms than vegetative cells. Trehalose content is likewise important and correlated with heat shock protein production, both of which are found at lower levels in growing vegetative cells compared to ascospores (Hottiger et al., 1989). Sclerotia and chlamydospores are often more heat resistant than growing hyphae and yeast cells, but are less frequently associated with commercial spoilage than ascospores.

Intra- and interspecies variability

Extreme variability among ascospore thermotolerance exists within and between species. The Trichocomaceae family contains the main HRM that plague the food industry, although other genera outside of this group, most notably ascospores of the model filamentous fungi *Neurospora,* have increased thermal resistance as well. Within the family Trichocomaceae, there exists a large degree of structural and biochemical diversity that contributes to the variability in thermal death kinetics (Conner and Beuchat, 1987a). The interspecific variation in solute content may provide a competitive advantage, depending on environmental conditions. The identity and ratios of soluble solutes vary be species, and within species by cell type and growth conditions. Mannitol is a superior cryoprotectant and contributes to osmotolerance, while trehalose provides increased thermostability and has a higher water binding

capacity (Wyatt et al., 2014). *Aspergillus* (*Neosartorya*) ascospores have more trehalose than ascospores of *Talaromyces,* although *Talaromyces* is the more heat tolerant. *Aspergillus* (*Neosartorya*) ascospores accumulate a greater diversity of sugars and sugar alcohols and are more resistant to desiccation, a strategy which may provide protection from fluctuating environmental water availabilities (Wyatt et al., 2015a). There are tradeoffs to cytoplasmic accumulation of compatible solutes. Lower solute concentrations decrease thermal resistant, but spore germinate more rapidly under supportive conditions (Judet Bensoussan et al 2008, Nanguy, Perrier-Cornet et al, 2010).

The most heat resistant genera of relevance to the food industry are *Paecilomyces* (*Byssochlamys*), *Aspergillus* (*Neosartorya*), and *Talaromyces* (Table 1.3). Other genera, however, are sufficiently heat resistant to cause spoilage within shelf-stable products. These include *Aspergillus* (*Eurotium*), which has been isolated from grape preserves and can survive 2.5 min at 70°C (Splittstoesser et al., 1989), and *Monascus,* which has been isolated from preserved olives and could survive 7 min of exposure at 75°C (Panagou, 2002). Moreover, variable heat tolerance is observed among the conidia of different strains, making them pertinent spoilage organisms in canned food when process deviations occur (Snyder, unpublished data). Samson et al. (2009) in a survey of *Paecilomyces* (*Byssochlamys*) isolates concluded that there are mesophilic, thermotolerant, and thermophilic individuals within the genus. The strain-to-strain variability published within the scientific literature may not accurately represent this diversity due to HRM isolation methods which select for high levels of heat resistance (Table 1.3). Within the published literature, *D*-values (time required for

a one log reduction in microbial count at a given temperature) for ascospores can vary by hundreds of minutes at 85°C (Rajashekhara et al., 2000). *Aspergillus* (*Eurotium*) is inactivated almost instantaneously while *Talaromyces macrosporous* and *Aspergillus* (*Neosartorya*) *fischeri* can survive for over 100 min, even longer if ascospores are contained within the fruiting body, although this varies by strain. Other *Aspergillus* (*Neosartorya*) isolates, along with many of the published values for *Paecilomyces* (*Byssochlamys*) have *D*-values on the order of 10 min at 85°C (Dijksterhuis, 2007). Conidia of *Aspergillus niger, Aspergillus* (*Eurotium*)*, Penicillia,* and *Wallemia* have *D*-values that range from 3 min to 230 minutes at temperatures from 54 to 62°C.

Organism	Temperature	Reported decimal reduction (D-values) in minutes		
		Neutral	High Acid	Low A_w
Paecilomyces (Byssochlamys)	85ºC	10[1] 12.7[1] 14.6[2]	4.5[1] 1.3[1] 12[3]	120[3] 35[5] 19[2]
Aspergillus (Neosartorya)	85ºC	30 [6] 35.3[7] 11.2[2]	13.2[6] 10[6] 14.5[5]	39[8] 26[9] 32[10]
Aspergillus (Eurotium)	70ºC	No Data	2.5[14] 2.9[11] 2.2[13]	5.2[14] 4.6[14] 17.2[2]
Talaromyces	90ºC	6.2[6] 2.0[9] 7.1[8]	5.2[8] 2.2[15] 2.9[15]	11.1[8] 11.7[11] 7.6[12]

Table 1.3: The physicochemical properties of food matrices impact the thermal inactivation kinetics for various heat resistant molds (HRM). The above thermal destruction curves approximate the survival of heat treated ascospores under various conditions. Strain-to-strain variability is illustrated by *D*-values for three strains of a given species.
1.Dijksterhuis (2007); 2. Kotzekidou (1997); 3. Splittstoesser and Splittstoesser (1977); 5. Casella et al. (1990); 6. Conner and Beuchat (1987b); 7. Rajashekhara et al. (1996); 8. King and Whitehand (1990); 9. King et al. (1969); 10. Worobo unpublished data; 11. Beuchat (1986); 12. Tournas et al. (1994); 13. Scott and Bernard (1987); 14. Splittstoesser et al. (1989)

Matrix effects

Survival is also influenced by the food matrix (Table 1.3). Although there is a great deal of strain-to-strain variability, general trends in matrix effects exist. Reduced water activity products, such as syrups and concentrates, provide a protective effect and inactivation is reduced. High acid matrices, like fruit juices, increase the efficacy of thermal inactivation. The combination of factors contributing to survival makes inactivation difficult to predict unilaterally, and it often requires process and product specific evaluation. *Aspergillus* (*Eurotium*) is a xerotolerant fungus, and the presence of increased extracellular solutes provides a protective effect (Wheeler and Hocking, 1988). Studies have found limited heat resistance among some isolates, and little if any data exist on ascospore survival in neutral conditions (Splittstoesser et al., 1989). The principle behind the protective effect exhibited in low water activity environments may involve relieving the disparity between the solute concentration in the medium and ascospore, and reducing osmotic pressure. In extreme water limiting environments, though, the benefit of protection may be mitigated by the inhibition the matrix imposes on germination. Although the ascospore survives the thermal process, it cannot grow in the food matrix which is preserved through water limitation. However, if the matrix will be diluted in a subsequent production step, as is the case for apple juice concentrate, survivors may germinate and cause spoilage.

Acidity contributes to more rapid reduction in the fungal population during processing. Benzoic, sorbic, citric, and acetic acid all decrease heat resistance compared to thermal inactivation curves generated in neutral conditions (Beuchat, 1988; Rajashekhara et al., 1998). As discussed in the previous section, the identity of the acid also influences the destruction process. *Aspergillus* (*Neosartorya*) spores were

inactivated more rapidly in grape juice than in apple (Conner and Beuchat, 1987a). *Aspergillus* (*Neosartorya*) counts were reduced by 1 log after 66 min in apple juice at 80°C, and 33 min in grape juice. *Aspergillus* (*Neosartorya*) and *Paecilomyces* (*Byssochlamys*) were inactivated more readily in cranberry juice than grape even though both matrices were adjusted to a pH of 2.5 (Dijksterhuis, 2007; Splittstoesser and Splittstoesser, 1977). Fumaric, sorbic, and benzoic were more lethal than acetic, malic, and citric, and tartaric acid at the same pH, although lethality increased generally as pH decreased (Beuchat, 1988). Generally, citric and acetic acids are regarded as the most effective organic acids, compared to tartaric and malic. However, these general principles are likewise dependent on the total composition of the matrix and are influenced by the presence of additional solutes.

1.4 Opportunism in the food production and processing environment

As a consequence of the structural and metabolic features described above, fungi are well adapted to proliferate within food production environments, in addition to the products themselves. Elements of the food processing infrastructure, from equipment and packaging materials to processing water and air circulation systems, have been associated with spoilage incidents in a variety of products. Many of these quality failures have resulted from poor sanitation practices, and were resolved through re-evaluation of sanitation and GMP (Good Manufacturing Practice) prerequisite programs. Monitoring records for visual inspections of cleanliness and tracking trends in total yeast and mold counts, or indicators, in the environment or finished product are useful in assessing the efficacy of these prerequisite programs. Aspects of manufacturing that may be important areas of focus for fungal contamination control

are discussed below.

Materials and Equipment

Fungi are harbored by materials and equipment commonly used in food production. Porous organic materials, known to be problematic, are still used extensively in secondary shipping containers, wooden pallets or crates for storage, and paperboard fillers and slip sheets. For those items which are re-used, cleaning and sanitation has limited efficacy. This has been observed in the apple industry which has traditionally relied on wooden bins during long periods of cold storage. Secondary apple pathogens which colonize the fruit after harvest and senescence, notably *Penicillium expansum* and *Paecilomyces* (*Byssochlamys*) spp., are transferred between seasons as the fungal population builds on the wooden surface, effectively inoculating subsequent lots (Okull, et al., 2006). Moreover, these fungi are known mycotoxin producers and wooden bins and crates can contribute to violative levels of patulin in the final product. Despite this, many orchards have invested in these systems and total elimination of wooden equipment may not be realistically feasible. Cardboard and paper materials used in packaging or shipping are also notorious harborage sites for fungal spores. Studies have shown that the greatest concentration of HRM in a production facility is around the depalletizer, and facilities with the space and resources physically separate this area from the rest of the food processing environment to prevent inoculation of exposed product with fungal contaminants (Rico, 2016). Finally, temporary or retrofitted equipment is often of poor design and integrates construction materials that are hard to clean and may harbor spoilage fungi. Although they may be utilized infrequently or only temporarily throughout the facility, using tape to temporarily affix or hold items in

place and the placement of cardboard slipsheets near doors to prevent drafts, under tables so they are level, or under employee workstations to provide cushion represent an unnecessary introduction of potential contaminants and are usually associated with the additional failure of another prerequisite program within the facility.

Other fungi are particularly associated with buildup on processing equipment made of stainless steel, plastic, or rubber. Notably, *Geotrichum candidum*, collects on fan blades, air vents, and rubber seals in food processing environments and is commonly known as "machinery mold" (Table 1.1). Resembling dust or dirt accumulation, *Geotrichum candidum* can cause flavor changes if permitted to grow in food products. Cleaning procedures targeting *Geotrichum candidum,* as with all sanitation programs, should consider the potential for cross-contamination. After cleaning surfaces contaminated with *Geotrichum candidum* (fan blades, air vents) that are adjacent to processing equipment, facilities may notice a temporary increase in airborne mold spores due to aerosolization and may consider the timing of product exposure consequently. Some species of filamentous fungi, along with yeast, are associated with the internal surfaces of processing equipment contacted by the product and are difficult to clean. These include ports, valves, filler heads, and gaskets that require disassembly to properly clean, and serve as barriers or interfaces between the product and the environment. Similarly, regions where proper circulation during processing and CIP are essential for sanitation, including potential dead legs and tubular heat exchangers are areas where biofilms can lead to product contamination (Lawlor et al., 2009). Yeast can contaminate the filler due to adhesion, and the potential for yeast biofilm formation and persistence in processing equipment is addressed through rigorous sanitation that is

appropriate for the product, potential spoilage organisms, and equipment design (Wang, 2015). Using heated CIP systems (Clean In Place) allows for treatment of adjacent surfaces, while specific cleaning solutions may be more or less useful on processing equipment that handles high levels of lipids, proteins, calcium, or that are high acid. Consultation with the equipment manufacturer and sanitizer provider will help ensure an appropriate strategy is selected.

Air and Water

Air and water movement throughout the facility serve effectively as vectors for dispersion of fungi. Air systems may be in place to maintain positive pressure over production, or air may be filtered through a HEPA system. Airborne molds can prove particularly challenging during filling, and many facilities choose to cover and contain their fill step, segregating it from the rest of production (Cook and Johnson, 2009). Lack of air pressure controls has been associated with fungal spoilage (Wang, 2015). Compressed air used to remove debris from packaging or stationary food contact surfaces should be considered for its contribution to microbial quality. Similarly, blast freezers which move air at high velocities have also been a source of product contamination (Cook and Johnson, 2009). Prolific conidia producers can be found ubiquitously in the environment. Reportedly, *Aspergillus, Cladosporium,* and *Penicillium* spores can be found in every cubic meter of air (Wyatt et al., 2013). The density of spores in an area varies seasonally, and are most concentrate during spring and summer (Adams, 1964). Mold spores constitute 95% of the aerial microflora over fruit orchards. The spread of aerosolized mold spores can be managed through various design and sanitation programs (Moritz and Martiny, 1997). As discussed, segregation

of depalletization/shipment from the production floor, and segregated and covered filling of the product into the final container can help reduce the potential for airborne fungal contamination. Segregation of dry or powdered ingredients during storage and when measuring out ingredients for processing can likewise help reduce the amount of air particulate (Cook and Johnson, 2009). Dry sanitation regimes have limited efficacy against fungi (Rico, 2016), and, in contrast, sanitation regimes like fogging may prove particularly efficacious at reducing the airborne mold spore load. Air quality systems should be monitored for efficacy. Again, consultation with the equipment manufacturer will help ensure appropriate monitoring techniques are employed. Activities may include direct plating of pressurized air, determination of filter change frequency, or quantitative air sampling.

Water is another medium which can transfer fungal propagules within a facility and water, more than air, is associated with yeast cross-contamination. Yeast are less likely to be introduced by air alone, but may be carried in aerosols. Contamination due to splash and overspray during sanitation programs are major vectors for the spread of spoilage yeasts. High levels of yeast can frequently be cultured from floor drains, and high pressure sprays during cleaning can splash contaminants onto processing equipment. For this reason, it is recommended that a final sanitizer rinse is applied to processing equipment after cleaning the floors. However, appropriate spray pressure can also reduce the introduction of spoilage organisms in the first place. Insects also serve as a significant source of yeast contamination and should be controlled in production facilities (Stratford et al., 2008). Insects are attracted to standing pools of water or spilled product which when not rapidly eliminated, and when poor quality or poorly

handled raw products are used, may also be attractants. Moisture should generally be limited within production facilities through air conditioning and temperature control. Condensation formed over production lines or in cooling units serves as a conduit for yeast cross-contamination. Similarly, proper cooling for finished products can help reduce condensation formation and spoilage organism ingress in products. Yeast spoilage has been observed in products where moisture has introduced contaminants from packaging and closures (Lawlor et al., 2009). Active cooling systems which use circulating water, such as cooling tunnels, cooling towers, or cooling sprays, can introduce spoilage organisms into finished product through this same mechanism. In this case, the microbial quality of the water supply should be managed since it is intentionally applied to cool the container. Although that water is not intended to reach food contact surfaces, the cooling food inside the container may draw in water as the vacuum forms, particularly if the incipient seal is disturbed, commonly referred to as "leaker spoilage" (Table 1.1). Similarly, pinhole or microcracks may form momentarily along seams during retort, introducing microscopic amounts of extraneous water (Black and Barach, 2015). For this reason, many processors have elected to use breakpoint chlorine in their processing water to inactivate potential contaminants. The organic load in processing water is greatly influenced by recirculation, and the frequency with which containers rupture, releasing product into the system.

Sanitation Programs

Lax sanitation regimes are responsible for most of the spoilage issues facing commercially processed food (Sperber, 2008). Without effective sanitation, fungal populations will build up within the production system and become enriched in spoilage

fungi that proliferate well on the food substrate. Dormancy in spore states also facilitates fungal persistence in processing environments since die off due to environmental exposure is limited. Without rapid and consistent elimination of harborage sites, persistors collect within the environment until conditions that are growth supporting facilitate their germination. In order to eliminate organic debris and fungal niches, sanitizer selection should be informed by the food composition and innate resistance of pertinent spoilage organisms. For example, yeast like *Zygosaccharomyces bailii* are resistant to many common sanitizers, so for processors concerned about yeast spoilage (e.g. juice manufacturers), appropriate chemicals which have demonstrated efficacy against the target microbe should be employed (Brul and Coote, 1999).

Employee practices are predictors of food quality. Employee practices have previously been associated with the incidents of HRM in raw agricultural commodities (Jesenska et al., 1992), and GMPs for employee health and hygiene reportedly influence microbial product quality. Yeast spoilage from *Candida* and *Hanseniaspora* in fruit juices increased dramatically in samples taken from the processing environment compared to environmental and fruit samples taken from the orchard (Wang et al., 2015). The authors speculate this increase in yeast prevalence is from the employees during harvest and processing, or more generally from yeast contamination within the built environment. Further testing revealed that while only 7.4% of the yeast strains isolated from the orchard were osmotolerant, 71% of the yeast isolates from the processing environment were osmotolerant. The availability of product-associated nutrients acts as a selective pressure limiting the diversity of pertinent spoilage fungi

within the facility and increasing the population of well-adapted microbes (Senses-Ergul and Ozbas, 2006; Wang et al., 2015; Pitt and Hocking, 2009).

1.5 Fungus prospectus

Continuing work in spoilage mycology will aid in the development of targeted food quality programs which reduce contamination and the growth of yeast and filamentous fungi. This work faces several technical barriers to root cause analysis, following a spoilage incident, and targeted process development, in the prevention of spoilage incidents. Morphological identification of fungi is tedious and time consuming, often not utilitarian enough for industrial applications. Many references suggest that morphological typing should include examination on multiple media and comparison of growth rates therein, as well as characterization of conidia formation, ascospore shape, and the presence or absence of chlamydospores through microscopic examination (Samson et al., 2009). Although this may provide useful information, it also requires a great deal of in-house knowledge concerning classical mycology. Molecular methods may be faster, although growth of a pure culture is still needed. However, utilization of ITS (Internal Transcribed Spacer) sequencing, the consensus barcode region for fungi, has only genus-level resolution which can minimize the utility of the findings. Amplification of additional regions can be used to identify the species, but this additional typing may again necessitate case-specific consideration to select appropriate secondary amplicons and require PCR optimization due to strain-to-strain variability. Similarly, reliable and universal subtyping methods, such as RAPD (Random Amplification of Polymorphic DNA) or allelic typing, to trace spoilage organisms back to point sources are lacking in both the food and medical mycological fields.

Additionally, phenotypic misleads, like the somewhat erroneous assumptions that oxygen limiting conditions completely control the growth of filamentous fungi, and that a spoilage mold isolated from a hot filled product is necessarily heat resistant, as described above, further complicate the ability of processors to perform root cause analysis. Rapid and sensitive identification methods would facilitate processors tracking and trending background mycobiota as part of a preventative quality control program.

Establishing reliable controls and prevention strategies has become increasingly important in contemporary food production. Many of the current consumer demands and distribution needs have elevated the risk of fungal spoilage in processed foods. The increased globalization of the food supply has led to longer transit times, which increases the risk for temperature abuse and the extended time often needed for fungal outgrowth. National and international transport also leads to complex traceability, a challenge which plagues the food industry and impacts more than just quality control. Consumers continue to demand minimal or non-thermally processed products for a variety of reasons, but reducing or eliminating thermal treatments increases susceptibility to spoilage and decreases shelf-life. At the same time, emerging non-thermal pathogen inactivation processes offer many advantages over traditional thermal treatments and should be considered in regard to fungal inactivation as they are commercialized. Target fungi will need to be selected in these exercises, and identification of the relevant spoilage organism and the use of food-relevant strains is essential in determining appropriate processing parameters. Dantigny et al. (2009) has previously noted that spores grown under laboratory conditions may not be representative of environmental contamination. Desiccation and domestication work to

increase and decrease, respectively, spore tolerance to various treatments. Consumers are likewise demanding changes to product formulation, including preservative elimination, sodium and sugar reduction, and the use of natural flavors which all have the potential to increase the threat of fungal spoilage. The trends in packaging that contribute to spoilage potential are related to sustainability goals and waste reduction. The switch to PET (Polyethylene tetrephthalate) packaging for shelf-stable beverages from glass permits the ingress of atmospheric oxygen (Lawlor et al., 2009), while decreases in plastic and can thickness increase the probability of container damage and can decrease the maximum fill temperature that the packaging can withstand, which likewise impacts the formation of a hermetic seal (Evancho et al., 2009). Additionally, recycled and re-use packaging programs may meet a growing interest in waste reduction. However, any re-use of these containers is a significant source of spoilage fungi particularly suited for growth within the product. Cleaning and sanitation programs for these containers should be rigorous, and performed in a separate location from the production area (Lawlor et al., 2009). Solutions to these and future challenges will need to consider the mechanisms fungi have developed to disperse, survive, and propagate in highly restrictive food-relevant environments.

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CHAPTER 2

The incidence and impact of microbial spoilage in the production of fruit and vegetable juices as reported by juice manufacturers

ABSTRACT

Microbial spoilage of fruit and vegetable juices represents an important threat to food quality and an area of concern in reducing food waste. Despite this, relatively little research is dedicated to microbial spoilage compared to other aspects of food microbiology. Establishing the incidence and impact of microbial spoilage in juice production would provide justification for future research. In this study, we present the findings from a survey of juice processor members of the U.S. based Juice Products Association and the European based International Fruit and Vegetable Juice Association (31.1% response rate). Respondents were asked a series of forced choice, Likert-type, and open response questions regarding microbial juice quality challenges and control measures regarding their facility. The vast majority of respondents (97.4%) indicated that spoilage mattered a lot or a great deal in brand protection. An additional 89.5% indicated that better control over microbial food spoilage would reduce waste and increase profits, with 57.9% indicating a lot or a great deal of impact, perhaps as a result of the frequency with which respondents indicated they discarded ingredients or product to protect quality. The most frequent disposition reportedly occurred on a weekly basis, with over half of respondents indicating discarding ingredients or product at least annually. Manufacturers reported a range of challenges, notably spoilage from *Alicyclobacillus* and heat resistant mold, and associated sanitation and production control strategies. This work provides a basis for subsequent research exploring

improved control strategies and detection methods used to reduce microbial spoilage of fruit and vegetable juices.

2.1 Introduction

Food waste and loss

Food quality issues resulting from microbial food spoilage are a significant problem within the food industry that result in waste, customer dissatisfaction, and that threaten brand protection. Global estimates on food waste and loss suggest that 40% of the food supply is not consumed due to pre-harvest loss or post-harvest food waste (FAO, 2012). Specifically, 25% of the post-harvest food supply may be wasted due to microbial food spoilage (Gram et al., 2002). Fresh produce and fruit and vegetable processed products are particularly susceptible to spoilage. The Food and Agriculture Organization (FAO) of the United Nations estimates that approximately 45% of produce that is grown is wasted (FAO, 2012).

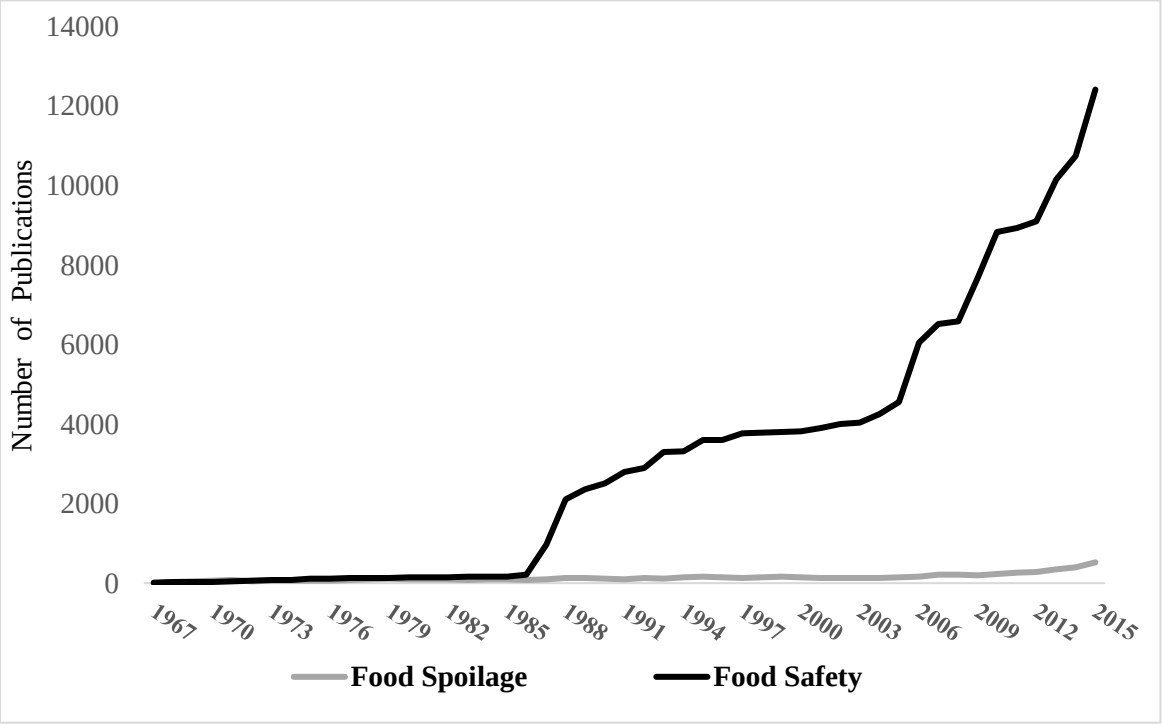


Figure 2.1: Publications by topic in the Web of Science food science resource database (FSTA). The number of annual abstracts containing the words "food safety" have increased dramatically since 1985 in comparison to the number of annual abstracts containing the words "food spoilage."

Historically, relatively little research has been conducted in the area of food quality/spoilage (Fig. 1). Food laws and regulations are focused on food safety and public health, as opposed to quality and microbial spoilage. In the U.S., exceptions include defect action levels associated with filth and/or fungal loads at levels which may indicate poor production practices (Title 21 CFR Part 117). Additionally, products subject to regulation under Title 21 CFR Part 113 and 114 must meet requirements for shelf-stability, an instance in which food quality, as it pertains to microbial food spoilage, is federally regulated (U.S. FDA, 2016a). Federal research dollars have, likewise, been directed towards reducing the incidence of foodborne illness, while the majority of available food security research has targeted pre-harvest loss specific to crop and food animal disease resistance and prevention (USDA, 2016). However, food waste occurs throughout the supply chain and in the developed world, waste is shifted towards the consumer end of the farm-to-fork spectrum. According to the FAO (2012), 40% of total waste is a result of waste in the hands of consumers in the United States. Increased global concerns involving feeding a growing world population and increasing sustainability have brought issues of post-harvest microbial food spoilage to the forefront.

Microbial food spoilage in fruits and vegetable products

Produce is often grown and handled in unprotected, natural environments which

contribute to the level and diversity of microbial contamination (Leff and Fierer, 2013). Although plants possess defense systems against microbial spoilage, following harvest and senescence, these defenses are diminished and the fruit or vegetable becomes particularly susceptible to colonization by secondary plant pathogens. This propensity for spoilage continues to increase as the produce is cut, pressed, or juiced and the intact surfaces of the fruit or vegetable are compromised (Barth et al., 2009). Subsequently, fresh produce tends to be highly perishable with a shelf-life as short as seven to ten days for products like fresh market raspberries harvested under the optimal conditions (Tezotto-Uliana et al., 2014). Fruits and vegetables used in juice production are a major source of spoilage microorganisms that can cause quality issues later in shelf-life, and the spoilage of these ingredients before processing contributes to waste within the industry, if they are discarded. Or, if the spoiled produce is utilized, it may cause increases in the initial microbial load and potential for post-production spoilage. Spoilage is defined as any change in quality that renders a product unappealing to consumers. This includes abiotic spoilage issues, like separation, enzymatic color changes, or moisture loss, in addition to biotic spoilage that results from the growth and metabolism of bacteria and fungi, yeast and molds, which can lead to turbidity, visible hyphal formation, off-flavors and aromas, and textural changes (Jos & Veld, 1996).

While the most obvious source of spoilage microorganisms is the production environment, contamination can occur throughout the supply chain (Zoellner et al., 2016). Initial microbial load can vary based on horticultural practices, employee health and hygiene, and aspects related to the field and surrounding areas. Older fields and orchards, or locations where dead and decaying plant material is not frequently

removed, can create harborages of spoilage microorganisms that result in a decreased shelf-life. The employees may be a source of potential spoilage microbes, like common yeasts that are found on the skin, but the transfer of these microbes depends on the adoption of hygienic practices including handwashing regularity and glove usage (Temelli et al., 2006). Agricultural husbandry practices impact microbial loads. Cultural practices which involve increased manipulation, the use of botanical amendments, and the lack of conventional pesticides increase initial microbial load at the point of harvest and are associated with organic production systems for fruits and vegetables. Additionally, the source of the agricultural water, use of particular growth substrates and insulation materials, and whether production environments are covered, enclosed, or open, all contribute to the potential for contamination.

At the point of harvest, the rigidity of the culling procedures can impact downstream spoilage potential. The utilization of drops, produce that has fallen to the ground, can introduce additional microorganisms. Similarly, the use of bruised or wounded fruits or vegetables can increase the potential for spoilage as wounds and lesions are niches where spoilage organisms may proliferate more readily than on intact surfaces, and may cause spoilage and loss of ingredients during storage prior to use in production (Snyder et al, 2016). Sanitation in the production environment is another important aspect in reducing the introduction of spoilage organisms into finished product. Organic material and vegetative waste that builds up on production lines can serve as reservoirs of spoilage organisms (Barth et al., 2009). Bottling or filling areas often require protection and treatment as critical quality points to prevent the introduction of environmental contaminants. Particularly in the case of mold spoilage

prevention, air quality monitoring can be essential as the spores of filamentous fungi often become airborne during the normal reproductive cycle to facilitate dissemination and propagation (Codina et al., 2008).

The relevant specific spoilage organism associated with a particular juice is dependent on the type of fruit or vegetable used as ingredients, and the type of processing the juice receives (Gram et al., 2002). Vegetables, generally, have a higher pH than many fruits which allows for the outgrowth of bacteria that would otherwise be selected against in fruit juices. Low acid vegetable juices are subject to spoilage from a variety of microorganisms which include the vegetable soft rotters like *Pseudomonas*, and various Enterobacteriaceae such as *Pectobacterium*, as well as bacterial sporeformers (Barth et al., 2009). High Pressure Processing (HPP) is a newly emerged technology that has gained a significant market share in the juice industry, particularly among specialty beverages, shakes, smoothies, and juices marketed as "lifestyle" products which include vegetable juice blends (Mintel, 2015). Juices produced using this process are sometimes referred to as "cold pressed" to emphasize the lack of thermal processing and benefits such as nutrient and flavor retention. HPP treatments reduce the microbial population and can extend the shelf-life of juices, but more resilient bacteria and fungi may survive the processing conditions established for a 5-log reduction in the pertinent pathogen.

Although non-thermal techniques like HPP have dramatically gained popularity among juice processors over the past decade, the majority of juice produced in the U.S. has undergone a thermal pasteurization (Mintel, 2009). Heat treatment, particularly at temperatures used in producing shelf-stable products, selects for heat resistant

ascospores from fungi like *Paecilomyces* (*Byssochlamys*) and *Aspergillus*, as well as bacterial spore formers (Splittstoesser, 1996). The additional selective pressure from acidic fruit juices renders the bacterial spore former *Alicyclobacillus* particularly problematic. This bacterium survives the thermal process as a spore, then germinates during shelf-life due to its acidophilic nature. Spoilage is characterized by the production of off-odors and aromas, namely guaiacol and other halophenolic compounds, that are described as smoky or medicinal. *Alicyclobacillus* spoilage is not associated with visual defect, the production of detectable off-flavors occurs well below the visual detection limit (Walker & Phillips, 2008). In contrast, heat resistant molds, or HRM, are associated primarily with visual defects. HRMs have posed a chronic challenge for juice producers as the heat resistant ascospores can be found as contaminants in the raw ingredients and the processing environment (Dijksterhuis, 2007; Tournas, 1994). Particularly with the growing popularity of plastic containers which lack a true hermetic seal, there has been an increased potential for spoilage incidents resulting from *Alicyclobacillus* and HRMs. Based on the history of challenges plaguing juice manufacturers, these two spoilage microorganisms were specifically addressed in this survey.

Available data on the impact of microbial spoilage in the food industry

Despite the contribution of microbial spoilage to waste and loss in the food supply, there remains a need for data establishing the contribution of microbial spoilage from the point of production through shelf-life. There are limited published data establishing the incidence and impact of microbial food spoilage on ingredient loss and finished

product spoilage. This is in contrast to food safety failures which become public record as a consequence of commercial recalls and outbreak investigations. In 2011 (Scallen et al.), the CDC published updated estimates on the incidence of food safety in the U.S., and this publication continues to be used extensively in the justification of food safety research. However, no such publication exists establishing the needs among manufacturers for better control over microbial food spoilage issues at the level of food processing and throughout shelf-life.

Based on the number of published abstracts relating to food science and technology, research focused on food safety has drastically increased since the mid-1980's (Fig. 1). This bibliometric assessment is based on literature containing specific search terms, and it should be noted that the term "food safety" was adopted around 1985 following the Listeriosis outbreak in California associated with soft, Mexican-style cheese (Linnan et al., 1988). Therefore, the 2,100 abstracts addressing food safety that were published in 1988 represents the initial baseline publication number based on the use of the term (Thomas Reuters, 2016). Well over 12,000 abstracts were published on food safety in 2015. In contrast, the number of abstract containing the term "food spoilage" has remained relatively stagnant, well below 500 abstracts per year from 1967 to 2012, with only a modest increase in publication number in the past few years. Despite this disparity in research publications, it is the premise of this study that there is a need for additional work in the area of spoilage within the food industry. Subsequently, a publication quantifying the impact of microbial spoilage on the food industry would contribute to the justification of additional food quality research.

Pettipher et al. (1997) determined that 14.7% of commercial orange juices were

contaminated with *Alicyclobacillus.* A previous study (Walls and Chuyate, 1998) surveyed members of the National Food Processors Association and determined that 35% of juice processors had experienced *Alicyclobacillus* spoilage of their product. These limited and outdated data do not reflect some of the changes and current trends in juice production that have influenced industry practices. As mentioned earlier, the use of plastic juice bottles for shelf-stable juices, instead of hot-filling into glass containers with a plastisol-lined metal lid, leads to a change in the headspace composition (Graumlich et al., 1986). And the use of thin-walled plastic containers popular for some products requires manufacturers to reduce the thermal process as these containers cannot withstand high temperatures. This is in addition to the demand among consumers for minimal processing, generally, contributing to the increased popularity in the cold-pressed juice market, and minimal or no use of food additives. Juices may also be used as ingredients in other foods and beverages, and are subsequently a considerable source of spoilage microorganisms in downstream products. Increasingly, the use of juice or other natural flavors have gained traction over the use of synthetic flavor additives. This change, however, represents an increased risk for spoilage as a consequence of substituting an ingredient with a lower propensity for harboring problematic spoilage organisms, for one with a higher propensity. Similarly, companies have sought to reduce sodium, sugar, and other ingredients with antimicrobial properties to meet consumer demands, affecting the stability of other products in which juice is used as an ingredient (Mintel, 2015).

Obtaining information on industrial spoilage incidents is difficult as there are no reportable food quality incidents, unless they have food safety implications as well or if

a product withdrawal is initiated. Among U.S. based class III (unlikely to cause illness or injurty) recalls from 2005, the average cost of a spoilage incident due to "mold contamination" or "fermentation" was $2.3 million (Lawlor et al., 2009). Individual companies have little impetus to share food quality challenges despite the need for aggregated data to justify a deserving research area. The lack of data establishing the total impact of microbial spoilage and establishing the organisms with the greatest spoilage potential leaves the food industry susceptible to ongoing quality issues. Despite this, companies may perceive food quality surveys as a potential threat to their brand, if they are asked to reveal information regarding quality defects or spoilage incidents, and they may feel that revealing their control strategies and sanitation practices is an infringement on proprietary information. Therefore, anonymous surveys which seek to capture the frequency and incidence of microbial food spoilage issues faced by manufacturers in an anonymous, brief, and non-judgmental format are likely to obtain higher response rates from a variety of manufacturers (Jespersen, 2016). The objective of this study is to present a quantitative assessment of the impact and incidence of microbial spoilage in fruit and vegetable juices as reported by manufacturers by using a survey tool that was iteratively developed through stakeholders in international professional associations of juice manufacturers.

2.2 Materials and methods

Survey group

Data were collected from two professional juice manufacturer associations between July 22nd to August 5th, 2016. The survey was administered to the Juice Products Association (JPA) based in the United States (120 companies) and the International

Fruit and Vegetable Juice Association (IFU) based in Europe (approximately 44 companies). In total, about 164 fruit and vegetable juice manufacturers (juice producers and/or bottlers) received the request for participation in this survey.

Survey tool development

Questions were initially developed based on relevant spoilage considerations as documented in the scientific literature. The prototype of the survey tool was sent to the Technical Affairs committee of the JPA (which included the coordinating IFU member) for review. Suggestions were integrated into the survey, which received a second and final approval from the committee. A description of the study, the benefits provided with participation (none), and anticipated risks (low risk, equivalent to every day internet use) were included on the first page of the survey. The tool was developed using the online platform, Qualtrics (Qualtrics, Provo, UT). Questions were presented as either forced choice (Yes, No, or Not Applicable, as needed), selection from a Likert-type scale, or open fields for discursive responses. Respondents were permitted to skip and return to all questions as desired. The survey tool was submitted along with an application for Institutional Review Board exemption to Cornell University's IRB office and exemption status was received on July 18th, 2016.

Questionnaire administration

They survey was delivered to participants through a link embedded in an email and distributed to the listservs for the JPA and IFU. Reminder emails were sent one week after the initial email and one day before the survey window closed. No compensation

was offered and participation was voluntary and anonymous. Survey responses were received from 51 participants (31.1% response rate). Responses were not linked with any identifiable information to protect anonymity.

Data analysis

The responses were collated through the Qualtrics user interface and exported to Excel (Microsoft Corporation, Redmond, WA). Examination of the data was performed using descriptive principles and tests (e.g. percent total of responses) to explore impact of various microbial spoilage challenges on juice production (Jespersen, 2016).

2.3 Results

Impact and significance of microbial food spoilage to juice manufacturers

The relative value of microbial spoilage in terms of its economic impact on fruit and vegetable juice manufacturers needed to be established. Participants were asked to assess the importance of maintaining product quality as it related to brand protection and dedication/conservation of resources through the selection of levels from a Likert-type scale (Fig. 2). Participants overwhelming (71.5%) responded that spoilage mattered a great deal in brand protection, while an additional 26.3% responded that it mattered a lot. The remainder indicated spoilage mattered a moderate amount in brand protection and no respondent indicated that spoilage mattered little or not at all. In terms of the value of dedicating resources towards preventing spoilage, 92.1% of participants indicated that spoilage merited either a great deal or a lot of consideration, and no participants indicated spoilage merited little or no consideration. When asked the extent

to which prevention of microbial spoilage would decrease waste and increase profits, 23.7% of respondents indicated it would have a great deal of impact, 34.2% indicated it would have a lot of impact, 31.6% indicated it would moderately impact the reduction of waste and increase of profit, while the remainder, 10.5% indicated it would have a little impact. Collectively, then, 89.5% of manufacturers indicated better control over microbial spoilage would have moderately to greatly increase profits and reduce waste. No participants indicated that better control over microbial food spoilage would not matter in terms of waste reduction and profit increases. Subsequently, the majority of fruit and vegetable juice manufacturers indicated that there was a significant need to control microbial spoilage as part of brand protection and that improved control over microbial spoilage both merits the dedication of resources and, in turn, would improve business efficiency by reducing waste and improving profits.

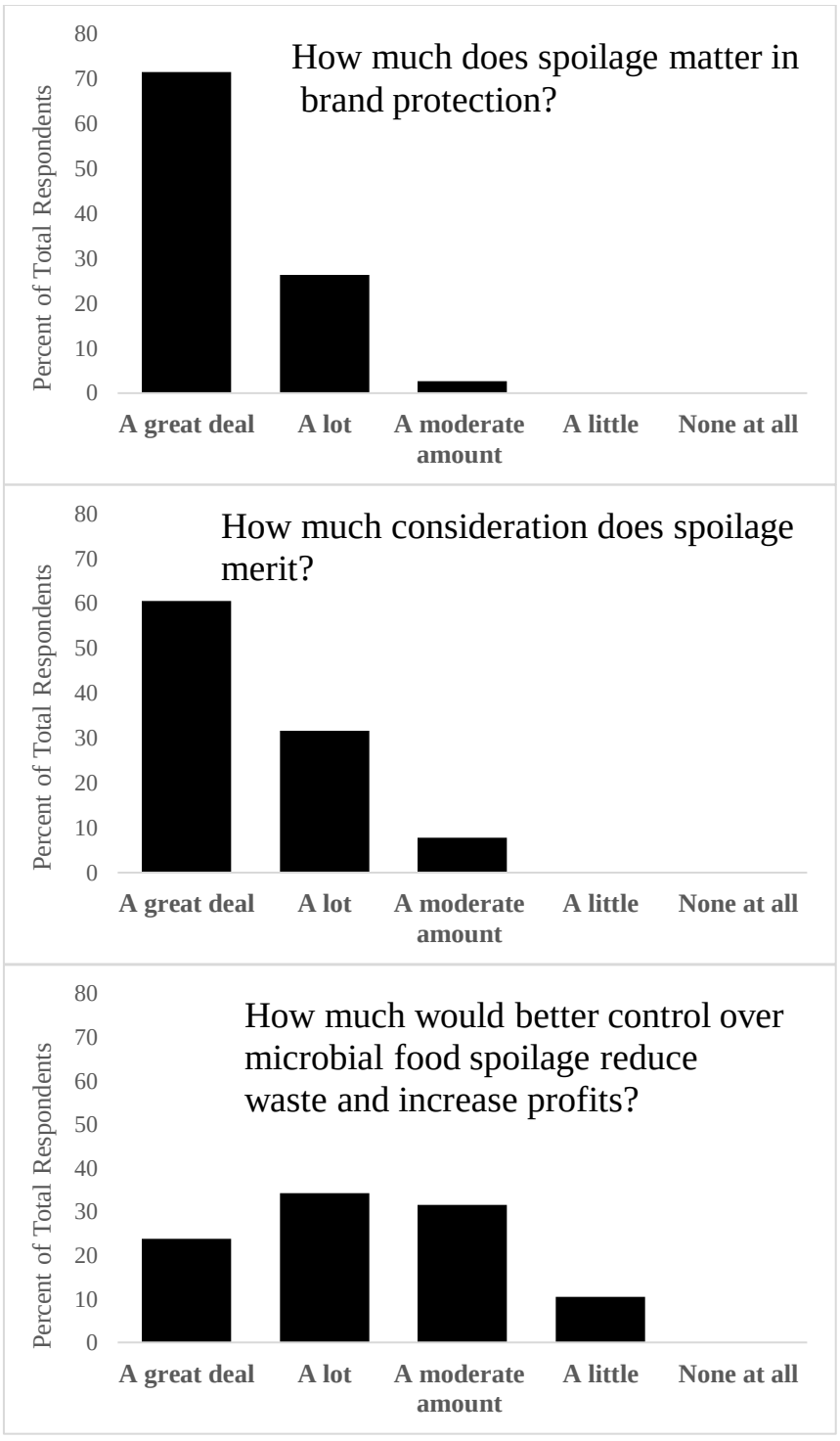


Figure 2.2: Self-reported impact of microbial food spoilage in fruit and vegetable juices. Results are reported as the percent of total respondents based on a Likert-type scale.

Problem areas and analytical techniques utilized by juice manufacturers

The perspective of industry stakeholders on the microbial spoilage issues in juice production would help guide research and development efforts to improve quality control strategies to address the areas of need established in section 3.1. Participants were asked a series of Yes or No questions regarding their experiences with particular types of spoilage (Fig. 3). In regards to fungal food spoilage, 89% of respondents had experienced mold or yeast spoilage in their ingredients and 92% of respondents had experienced mold or yeast spoilage in their finished product. More specifically, 64% of participants indicated that had experienced HRM in their finished product, indicating that HRM, as a category, are highly problematic for juice processers. As a consequence, 75% of participants responded that they utilized ingredient or finished product testing for HRM. Patulin, a mycotoxin produced by molds including *Penicillium expansium* and *Paecilomyces* (*Byssochlamys*) spp. is considered a chemical hazard in the production of apple juice (Daniels, unpublished data). Apples with core rot or blue/green mold spoilage may be culled to help control this hazard. Of the respondents who manufacture apple juice, 67% reported discarding ingredients or finished product as a patulin control. Finally, 78% of juice processors reported being concerned about *Alicyclobacillus* contamination. Overall, the findings generated from these survey questions regarding the problematic nature of particular spoilage organisms reveal that issues like fungal spoilage, particularly HRM, and *Alicyclobacillus* are not challenges faced by a few manufacturers. Even if spoilage issues arise sporadically, across the industry the majority of manufacturers face these challenged and are actively trying to manage the risks to their products' quality.

Question	"Yes" Responses	"No" Responses
Is *Alicyclobacillus* contamination a concern for your company?	78%	22%
Have you ever experienced heat resistant mold in your finished product?	64%	37%
Do you utilize ingredients or finished product testing for heat resistant molds?	75%	25%
If you manufacture apple juice products, have you had to discard ingredients or finished product to control patulin?	67%	33%
Have you had to discard ingredients or product due to spoilage in the past year?	69%	31%

Table 2.1: Incidence of bacterial and fungal spoilage and intervention strategies used in the production of fruit and vegetable juices. Results are reported as the percent of total respondents, excluding answers of "N/A" for product specific questions.

Food waste due to the quality deterioration of ingredients or finished product

The frequency with which manufacturers discard ingredients or product as a consequence of microbial quality deterioration is an indicator of the impacts that spoilage control strategies may have on reducing food waste and increasing food security. Juice manufacturers were asked to rate on a Likert-like scale the frequency with which they had to discard ingredients or products due to quality concerns. A total

of 16.2% of respondents reported discarding ingredients or finished products on no less than a monthly basis due to quality concerns. An additional 40.5% of respondents reported discarding ingredients or product on an annual basis, and 16.2% of respondents answered they discarded ingredients or products every few years. A total of 27% of respondents indicated they rarely or never discarded ingredients or finished product due to quality deterioration.

Control strategies utilized by juice manufacturers against spoilage microorganisms

In addition to ingredient quality, sanitation programs are another primary method manufacturers use to manage their food spoilage risks. When juice processors were asked if their sanitation program completely controlled contamination from spoilage microorganisms, 53% of respondents indicated that it did not. Additionally, 69% of respondents indicated that additional, targeted sanitation strategies designed to reduce harborages of spoilage molds would be useful to their company. Manufacturers reported a range of sanitation programs in place at their own facilities designed to reduce food spoilage issues. These included institutionalized food quality programs like SSOPs (Sanitation Standard Operating Procedures) and HACCP-based (Hazard Analysis Critical Control Point) plans, CIP/COP sanitation regimes and sanitation verification methods including ATP swabs, microbiological testing, and internal audits. Other manufacturers reported adopting mitigation strategies such as strict maintenance of the cold chain or elevated processing temperatures designed to target spoilage microorganisms.

Respondents were asked what additional work they felt should be addressed in the

area of food spoilage, and there were several independent discursive responses that dealt with particular aspects of HRM and *Alicyclobacillus* spoilage. These included thermal tolerance values, for both organisms, in different food matrices (e.g. high soluble solids, Brix), improved detection methods, and prevention from initial contamination. These two microbial spoilage issues received the most repeated comments about the need for additional research. Some respondents had very particular needs for the scientific research, including an improved understanding of the off-flavors produced by *Alicyclobacillus* and formulation strategies which may be used to control spoilage. However, other areas were addressed sporadically and included issues like biofilm elimination, resistance parameters of yeast to HPP, spoilage prevention in UHT products, anaerobic spores, and lactic acid bacteria. There were several mentions of non-microbial spoilage issues such as haze development in pear juice, adulteration with water or other beverages, and chemical hazards such as mycotoxins and plasticizers. Additionally, some manufacturers voiced concerns over the potential intersection of food spoilage and food safety issues. There also appeared to be a demand for improved risk management for microbial food spoilage. Respondents requested additional mitigation strategies, such as defined rework practices in the hot-fill industry, validation if CIP/COP methods, systematic prevention of cross-contamination, and sanitary controls for cooling water used in production.

2.4 Results

Brand protection as a function of microbial quality

Overall, the results of the survey indicated that there is a demand among companies

in the fruit and vegetable juice industry for increased control over microbial food spoilage. The preponderance of respondents who indicated that increased control would help in brand protection (Fig. 1) and have positive economic impacts (Fig. 1 and Fig. 4) may explain this response. There are numerous, recent commercial examples of food quality failures resulting in negative press for food companies. In 2013, a recall of Greek yogurt received national media attention (U.S. FDA, 2013). The product was contaminated with *Mucor circinellloides* which was later determined to be a post-processing contaminant (Snyder, 2016). Several hundred consumers reported gastroenteritis, prompting the recall. Indeed, respondents in this survey reported concerns about the intersection of food safety and food quality in the discursive portions. There are few examples in the literature of immunocompromised individuals contracting fungal infections as a consequence of foodborne fungal contaminants (Benedict et al., 2016), but those case reports are limited by their infrequency and the lack of culture-dependent of independent methods of traceback analysis. Most case reports are anecdotal or speculative. There are examples, however, of the metabolism of spoilage organisms creating microenvironments that allow for the outgrowth of bacterial pathogens, such as organic acid consumption by molds leading to bacterial growth (Wells & Uota, 1969). The propensity of spoilage to cause, directly or indirectly, food safety concerns is little understood and not well established in the literature. However, the inability of consumers to distinguish between food safety and food quality failures is well documented. Spoilage can be perceived, often correctly, as negligence on the part of the manufacturers in maintaining appropriate sanitation practices or processing conditions. However, the visibility of spoilage, like the visibility

of poor sanitation habits, attracts attention that is not necessarily proportional to the increase in food safety risk. Consumers may also report feeling ill when they recognize and ignore a quality failure as a consequence of a psychosomatic response that is not tied to a defined disease manifestation.

Impact of recent spoilage incidents on reported problem areas

Other examples of consumers reporting gastroenteritis associated with spoiled products include the June, 2016 recall of protein beverages (U.S. FDA, 2016b). This recall affected 3.8 million bottles which had quality defects impacting flavor, aroma, and texture. Similarly, over a dozen SKUs of a second UHT processed, protein beverage were recalled in June, 2016 "out of an abundance of caution" and as a result of quality defects that included bloated containers, and off flavors and aromas (U.S. FDA, 2016c). Spoilage by anaerobic spore formers and spoilage of UHT products were both mentioned in the responses from participants about areas of remaining concern, and the effect of these recent recalls may have influenced responses. However, spoilage of low acid juices and beverages by spore formers are, potentially, becoming increasingly relevant as the popularity of vegetable juices increases and various juice-containing dairy and nut milks increases. "Packaging defects" were identified as the causative issue in several of these company announcements including the March, 2016 recall of two batches of fruit and vegetable purees due to bloated pouches, off odors and aromas (U.S. FDA, 2016d). Three consumers reported instances of gastroenteritis but these, as in the other cases, were not confirmed to be related to the product. In 2012 and 2013, consumers reported mold growth in beverage pouches identified by mycelium

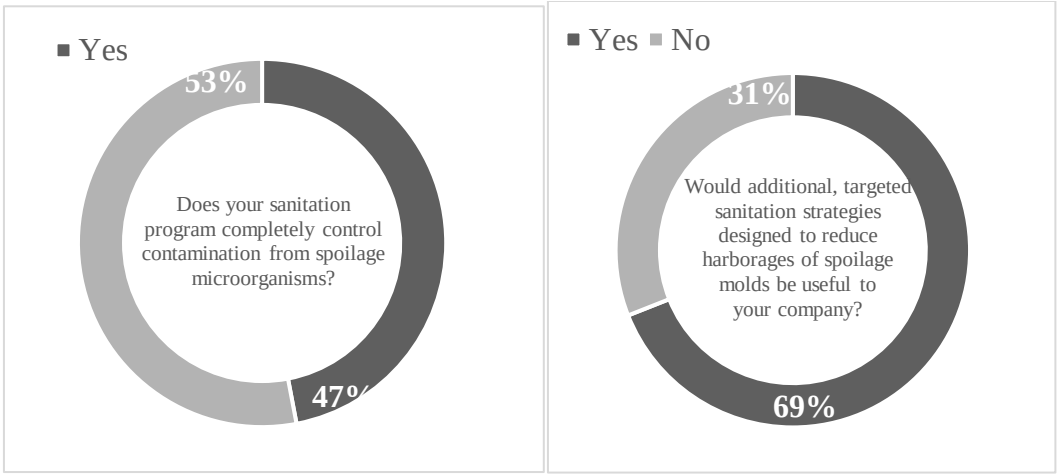
development that either clogged the straw or became visible once the package was cut open (Wong, 2014). A consumer initially reported finding a worm in their product which was later determined to be mold which had taken the shape of the straw. Juice and beverage processors report similar complaints by consumers who have misidentified mold in their product, so customer complaints must be closely evaluate. As a consequence of these spoilage events, the company has since opted to use a clear panel on their package so that consumers can see that their product is free from mold.

Figure 2.4: Food waste due to quality deterioration among fruit and vegetable juice manufacturers. Results are reported as the percent of total respondents based on a Likert-type scale.

The survey results and these recent food spoilage incidents reveal commonalities among the general responses of processors to handling microbial food spoilage. The importance of controlling food quality threats in brand protection (Fig. 1) is mirrored in the company announcements for their spoilage-based recalls. Several of the previous

announcements mention “defective packaging” as the reason behind the quality failure. Interestingly, package failure has not been reported as the root cause of these spoilage incidents and, furthermore, package integrity and sanitation is not mentioned by the manufacturers surveyed in this work as one of the risk mitigation strategies employed in controlling microbial spoilage (Fig. 5). Potentially, citing package failure may be a minimization technique that takes the emphasis off the food itself, and may be also a kind of generalization employed before the root cause is effectively determined. “Package defect” may also serve as a proxy for the fill step in production, which is sensitive to the introduction of spoilage organisms and may be considered a critical quality point among juice and beverage manufacturers. Another common phrase in these company announcements are the described quality defects by which consumers can recognize the spoiled product which repeatedly reference “container bloating and off flavors or aromas.” The visible development of mold is not usually cited, perhaps because of the negative associations consumers may have of such a visible quality defect, and one which may occur after container bloating. Visibly defective products are often the only spoilage-specific issues that rise to the level of product withdraws. For example, *Alicyclobacillus* spoilage is not associated with visible defect as the microbial count necessary to generate detectable off-aromas is well below visible detection. Moreover, consumer sensitivity to these off-aromas is highly variable, so that there is not a well-established limit at which consumers reject the product. Therefore, product withdraws due to juice spoilage as a consequence of *Alicyclobacillus* spoilage are unusual, no associated withdraws are known to the authors. Instead, *Alicyclobacillus* is a chronic quality defect that plagues the industry, but the problem is not well quantified

in the literature.



(B) Reported sanitation programs and monitoring practices				
SSOPs	ATP swabbing	Microbiological testing	CIP/COP	Ingredient inspection and grading
Antimicrobial treatment	Verification strategies including audits	Strict maintenance of the cold chain	Food safety programs (e.g. HACCP)	Elevated thermal processes

Figure 2.5: Value of sanitation strategies and analytical technique used as controls in microbial *food spoilage.* Results are reported as (A) the percent of total respondents and (B) discursive responses provided to the question "What sanitation programs and monitoring practices do you use to reduce the potential for microbial food spoilage?"

Current practices and areas for improvement in quality protection

The respondents indicated a discrepancy between the criteria established in processing and sanitation SOPs, as part of industry best practices developed to target microbial pathogens, and more rigorous criteria needed to control for spoilage microbiota. This response suggested that developing protocols designed targeting more resistant spoilage microorganisms may be an effective method for some manufactures to improve product quality and reduce the risk of spoilage. Conditions established for clean-in-place protocols and thermal processing, among other processes, are a function

of the target organism, and its relative resistance, used in challenge studies. Previous work has indicated the variability in phenotypic attributes regarding survival and proliferative ability among various isolates, emphasizing the importance of strain selection or cocktail composition in challenge studies. Additionally, strains isolated from particular environments have been shown to have an increased capacity for colonizing or spoiling particular food matrices. Adaptation of strains to matrix conditions has also provides an increase in microbial resistance. Bacterial and fungal strains which have been adapted to low pH, low water activity, and elevated or depressed temperatures have been shown to provide increased resistance to various treatments in juice (Usaga et al., 2014). Without proper isolate selection and adaptation practices, criteria (temperature, time, pH, type of acid, etc.) cannot be effectively validated in challenge studies. One respondent indicated a need for "good" thermal process values to control for *Paecelomyces* (*Byssochlamys*), another respondent indicated a desire for thermal destruction parameters (*D*, z, and F-values) for *Alicyclobacillus* spores, a third requested inactivation data for spoilage yeast treated with HPP processing, and a fourth requested CIP treatments validated against more resilient spoilage organisms.

This lack of specific messaging around industry best practices to control microbial spoilage hazards puts the burden on individual manufacturers to develop control strategies. Multiple respondents indicated that they had developed company and product-specific sanitation and processing approaches to control for microbial food spoilage (Fig. 3 and Fig. 5). These practices included in-house data-based decisions regarding the placement of critical quality points in their food quality plan, the

development of elevated processing or hurdle approaches, rigorous sanitation programs, and the use of produce sanitizers and preservatives (Fig. 5). Determining these practices can be excessively burdensome to smaller producers, and may be limited by the ability of manufacturers to collect data effectively. Many sanitation practices have been developed through the lens of controlling contamination from bacterial pathogens, with limited consideration for contamination from sources that are associated with transmission of fungi. Filamentous fungi produce conidiospores which are rapidly transmitted via air (Benedict et al., 2016). Although bacteria may aerosolize, fungal spores are evolved for air transmission to facilitate propagation in new locations. The ecological differences between bacterial pathogens and spoilage organisms also necessitates a change in thinking regarding master sanitation schedules. Locations which are not as commonly considered, including air vents, tops of fan blades, and other locations where dust and fungal spores can accumulate, may never receive dedicated sanitation efforts. When sanitation practices for these locations are initiated, consideration for the cross-contamination of adjacent locations must be considered. Industry best practices include cleaning food contact surfaces after cleaning non-contact surfaces, like floors, to prevent cross-contamination. Additional consideration may be required when cleaning the aforementioned surfaces where spoilage biota can accumulate. The aresolization of fungal spores during cleaning may be a significant consideration in preventing cross-contamination. Similarly, the sanitation of process water should also be considered from a quality perspective, and environmental monitoring procedures and detection methods need to be optimized and validated against spoilage microorganisms. Sampling locations and indicator organisms in

quality-centric environmental monitoring programs likely differ from those targeted in *Listeria* spp. environmental monitoring.

Tradeoffs and impacts

Although improvement in food quality is often presented as having positive impacts regarding sustainability and reducing food waste, one respondent pointed to the need for increased research on the tradeoffs between more intense sanitation programs and environmental impacts. The commenter specifically mentioned the impact of releasing waste water containing cleaning and sanitizing agents (salts, caustic or acidic solutions, detergents, etc.). Indeed, there are scant recommendations that are harmonized between the U.S. EPA guidance and GMP/GAP guidance for producers (U.S. EPA, 1999). And, as producers seek to increase the efficacy of their sanitization procedures by adding or increasing sanitizer levels, they also increase the potential to incur negative environmental impacts. Another respondent mentioned using a sanitizer in their flume tank to reduce microbial loads and, similarly, the addition and elevated levels of sanitizer at this step may constitute a tradeoff in environmental considerations.

By extension, rigorous cull programs to prevent elevated microbial loads through the addition of low quality produce, as discussed in section 1.2, may also increase food waste. Introduction of such practices may necessitate a diverted use for the culled produce; although, produce destined for juice, like other processed products, may already represent fruits and vegetables which have been culled from fresh market sale. The data presented in Fig. 4 illustrated the range in frequency with which respondents reported discarding ingredients or product due to quality, ranging from weekly to never

with a normal distribution, but these findings may underlie the responses in Fig. 2 regarding the economic benefit to improving control over microbial spoilage. Even manufacturers who rarely or never discard ingredients or products as a result of quality deterioration may only be able to do so at the cost of a quality program or strict supplier guarantees that, likewise, increase cost and waste at other points in the supply chain. Some respondents indicated a desire for processing conditions designed to target more resistant spoilage organisms. While these processes may have non-microbial quality tradeoffs that result from the processing treatment, these approaches may minimize microbial spoilage with less environmental impact.

Based on the responses of juice manufacturers to questions regarding the incidence and their intervention strategies (Fig. 3), the presence of *Alicyclobacillus* and HRM remains a serious threat to juice quality. Per the discursive responses, manufacturers requested improved detection, sanitation, and inactivation strategies against these organisms. Although these microbiological challenges have been known for over 50 years, manufacturers still report remaining needs addressing these problem areas. The data presented in this paper substantiated the need for continued and increased work in the area of microbial food spoilage.

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CHAPTER 3

Fungal spoilage ecology: Association of fungal genera with processed foods and production failures

ABSTRACT

Fungal spoilage in commercially processed foods causes consumer dissatisfaction and contributes to food waste. Many fungi, yeast and filamentous, are highly resistant to conventional processing controls used in food production. However, previous reports have suggested that these processing parameters and physicochemical properties create highly selective niches which typically support the growth of a limited number of spoilage organisms. In this study, the associated spoilage organism was identified from 127 commercially spoiled products through amplification and sequencing of the ITS region. Although these observations included a diversity of foods, the collection was overrepresented by juices and hot filled products due to the specialization of the research group. The prevalence and diversity of the identified spoilage fungi were evaluated in relationship to product specific attributes. Filamentous fungi, both heat resistant and heat sensitive, were isolated from hot filled products. Yeast were associated with spoilage of high acid, pasteurized or non-thermally processed products. *Penicillium, Aspergillus,* and *Cladosporium* were isolated in abundance across product categories, processing regimes, and physicochemical conditions. Spoilage fungi were partitioned into categories using this data, which may be adaptable for manufacturers as a decision tree for predicting spoilage organisms pertinent to individualized production systems, particularly manufacturers of juice and

hot filled products. The probabilities associated with this prediction model indicated that, in addition to formulation and processing conditions, the most common production failures associated with a given product may also inform identification of the specific spoilage organism. Identification of specific spoilage organisms, and the processing failures associated with their introduction and proliferation, can help processors develop better validation studies and control strategies.

3.1 Introduction

Fungal food spoilage

The select members of the fungal kingdom which are capable of contaminating and proliferating in commercially processed foods cause significant economic and security challenges for manufacturers, from shrink to consumer dissatisfaction. Approximately 25% of food waste and loss in North America is due to microbial spoilage (Gram et al., 2002), and fungi represent the most important group of spoilage microbes responsible for these losses (Sperber, 2009). It is estimated that fungi are responsible for 5-10% of all food waste and loss in developing countries, comprehensive of abiotic and biotic causes (Pitt and Hocking, 2009). Filamentous fungi, commonly referred to as "molds" within the industry, are the most common food spoilage microbe throughout the supply chain, across food sectors, and are even associated with spoilage of highly processed, high stability products (Criado et al., 2005). However, the specific spoilage microorganism, bacterial or fungal, responsible for the spoilage of raw ingredients or finished product depends on a combination of physical and chemical environmental factors.

Fungi are prolific food spoilage organisms as the majority of fungal species are saprobic and well adapted to nutrient derivation from non-living organic matter. These fungi are chemoheterotrophic and each express a suite of extracellular enzymes that, collectively in a complex ecosystem, are capable of digesting structural organic polymers during vegetative decomposition. Fungi function similarly in food products. However, classical fungal ecology often evaluates decomposition and nutrient dynamics in complex polycultures, whereas in food processed for stability and shelf-life extension, the microbial community is greatly depleted which, in concert with resource use efficiency, increases the probability of spoilage dominated by a single best competitor (Setala and McLean, 2004). It has subsequently been recognized that only a few microorganisms are capable of sufficient proliferative efficiency to become the dominant spoilage organism in a given system (Mossel and Ingram, 1955; Thompson, 2009). The association of specific spoilage fungi with particular food products and processes is essential for the advancement of quality control strategies (Pitt and Hocking, 2009). Identification of specific spoilage fungi has improved greatly since the 1990's due to the advent of molecular methods and international taxonomic consensus. The identification of a limited subset of fungi chiefly responsible for food spoilage in a specific product enables the development of targeted prevention and intervention strategies which reduce food waste and protect quality (Filtenborg et al., 1996).

Food spoilage is a sensorially detectable and can be recognized by a variety of different quality defects including visible mycelium formation, off-flavors and aromas, turbidity in liquid products, gas production, structural deterioration, exudate production, and discoloration. Fungal exoenzymes can directly cause defects such as the

development of off-flavors and the structural breakdown of the food matrix, and indirectly cause defects such as visible mycelium development and turbidity as they facilitate nutrient acquisition and support microbial proliferation. Various filamentous fungi secrete lipases, proteases, and carbohydrases which are highly specialized for a given environmental niche (Archer and Peberdy, 1997). The sensory threshold for some off-aromas caused by fungal catabolism can be quite low, as is the case with volatiles produced in the breakdown of sorbate by some Penicillia spp. (Larsen and Frisvad, 1995). Similarly, determining the fungal cell count which delimits or predicts spoilage is also challenging, due as much to the inherent problems in establishing quantitative cutoffs as it is to the difficulties in accurate enumeration of filamentous fungi. Establishing proliferative ability in a food product is essential for identification of the specific spoilage organism. Samapundo (2016) identified the limitations of previous studies on heat resistant molds (HRM) which focused exclusively on spore survival with no reference to mycelial outgrowth, which is often the characterizing defect in spoilage. Filtenborg et al (1996) likewise recognized the difference between isolated species which are present as propagules in food products or their processing environments, and isolated species which are able to spoil the food due to growth. Propagules, conidia, ascospores, and mycelial fragments, may be present as part of the environmental or ingredient-associated mycobiota without posing a quality risk to the product.

Processed foods as an ecological niche

Processing regimes, ingredient formulation, and pre-requisite programs (Good

Manufacturing Practices, Sanitation Standard Operating Procedures) are strategically implemented to optimize organoleptic quality and minimize microbial growth. Whole agricultural commodities possess microbial resistance mechanisms, which are reduced by senescence and further diminished by processing which violates structural barriers (Barth et al., 2009). Additional fabrication increases the downstream possibility for introducing environmental contaminants (Barth et al., 2009). This risk is mitigated in many products through thermal and non-thermal processing steps which inactivate microbial cells, and high acid and/or low water activity formulations which inhibit growth. Packaging conditions which limit the availability of oxygen also serve to impede the growth of filamentous fungi and aerobic bacteria. However, the diversity of spoilage fungi which are variably able to grow under extreme environments makes these organisms important targets in the development of control strategies and the likely cause of quality failure when deviations occur.

Although oxygen limitation is used to control the growth of filamentous fungi, *Byssochlamys fulva* and *B. nivea, Penicillium expansium* and *P. roqueforti, Geotrichum,* and *Xeromyces bisporus* are capable of growth with less than 3% oxygen tension (Pitt and Hocking, 2009; Scholte et al., 2004). In hot filled, shelf-stable products, vacuum is employed as a mold inhibitor, but some spoilage fungi have overcome this challenge due to the availability of residual oxygen. This risk is elevated in products with weaker vacuums and slightly higher total package oxygen due to lower fill temperatures, viscous matrixes, and packages which lack hermetic seals. Acid and water activity are conventional formulation controls which generally inhibit microbial growth, but fungal growth occurs over a wider range of physicochemical conditions than does growth of

most spoilage-relevant bacteria. Fungi grow in food products from pH <2 (organic acids) to >9 (mineral waters) and water activities from 0.61 to 0.99. In fact, several fungi can utilize organic acids as carbon sources, subsequently increasing the pH of the product, promoting bacterial growth. Weak acid preservatives, benzoate and sorbate, can also be broken down by fungi and their use selects for intrinsically resistant species (Rico, 2016). Osmotolerant/-phyllic and xerotolerant/-phyllic yeast and filamentous fungi abound and delimit the biological extremes for proliferation in water activity controlled environments. The identity of the solute impacts the ability of a given species to replicate. Yeast are better able to spoil high sugar solutions, compared to high salt or desiccated products of the same water activity. *Zygosaccharomyces bailii*, a common spoilage yeast, is somewhat nutritionally fastidious and can only utilize certain pentose and hexoses and requires a supplemental nitrogen source decoupled from its carbon source. Filamentous fungi have been recovered from sea salt and *Xeromyces* can replicate in media with a water activity as low as 0.61 (Grant, 2004). Filamentous fungi are also known for their ability to withstand temperature extremes. The sexual spores of some ascomycetes tolerate extreme thermal processing, surviving even the conditions used in generating high acid, shelf-stable foods that are "commercially sterile," or so called. Additionally, the refrigeration temperatures used to limit the growth of most microorganisms select for psychrotolerant bacteria and fungi. The restrictive growth conditions employed in food production impede the native microbiota, if they survive processing. The near-barren nature of these products allows those microbial contaminants which can replicate, to replicate without facing a high level of competition.

As a consequence of these highly selective conditions, often only one or a few microbes are able to contaminate and replicate to cause spoilage in a given product. These organisms have variably been referred to as the "critical fungi" (Filtenborg et al., 1996), the "associated microbes", or the "specific spoilage organisms" (Manios et al., 2014) once the limited spectrum of product-specific spoilage microbes became increasingly apparent as classical morphological identification was supplanted by molecular typing (Frisvad and Filtenborg, 1993). Even in less restrictive food systems (high pH and water activity, minimal thermal processing) like fluid milk and raw meat, spoilage is associated with the accession of a single group of best competitors (Doll et al., 2017). In raw and minimally processed foods with a diverse initial microbial profile, organoleptic spoilage is accompanied by decreased species richness and evenness. Interestingly, Fougy et al. (2016) observed that the microbial population in a less selective formulation of raw pork sausage, one with reduced NaCl, decreased in overall diversity more quickly than in a formulation with higher NaCl levels. A low level of salt may enable a subpopulation to outcompete other species more rapidly and lead to the rapid onset of quality deterioration with the elevated production of spoilage-associated volatiles. The expression of enzymes highly associated with nutrient degradation and off-flavors are not always highly expressed at low cell densities but are regulated through quorum sensing (Sperber, 2009). This may originate in the ecological role of many saprobes seeking to avoid triggering plant defenses and responses from competitor species. However, in processed products which utilize a greater number of hurdle technologies, the selective process is even more pronounced which increases the probability of a single spoilage organism.

Crucially, the ability to recover a microbe from a food product alone does not represent the ability of the microbe to spoil the product. Spoilage in commercially processed foods can result from a variety of production failures including under processing, post-processing contamination, problematic raw ingredients, process and formulation deviations, poor sanitation procedures, and improper hygienic design. Identification of both the associated spoilage fungi and likely production failure scenarios specific to a given product enable the development of targeted critical quality point assignment, focused monitoring strategies, and rapid troubleshooting. In this study, spoilage isolates were collected from commercially processed food products, along with data on the intrinsic and extrinsic formulation and process strategies, in order to determine product and process specific information predictive of specific spoilage fungi.

3.2 Materials and methods

Microbially spoiled commercial food products

In the present study, 127 microbial spoilage isolates were obtained from as many spoiled products submitted for evaluation through the food microbiology extension programs offered at Cornell University. All products were commercially processed, and raw or minimally processed (e.g. fluid milk) agriculture products were not considered. Products were manufactured by >50 different companies, primarily located in New York state. Products were grouped into nine product categories: juice/acidic beverage (n51), fermented (n12), baked good (n6), fruit preserve (n9), dried cereal and nuts (n8), confection (n13), refrigerated RTE (n18), butter and oils (n6), and tomato-based sauce

(n4), as similarly described by Filtenborg et al. (1996). Thermally processed fruit and vegetable products are overrepresented in this collection based on the specialization of the food microbiology extension program. Subsequently, 40% of the products belonged to the "juices/acidic beverage" category and 36% of the collection was manufactured using hot fill. The juice/acidic beverage category contained 100% juices, juice blends, and other acidic beverages which were either subjected to a kill step (thermal pasteurization, ultraviolet light, and high pressure processing) and refrigerated or hot-filled/bottle pasteurized and shelf-stable. The baked good, dried cereal and nuts, confection, and butter and oils categories were all thermally processed and shelf-stable due to sufficiently low water activity. The fruit preserve and tomato-based sauces (i.e. marinara, salsa, taco sauce) categories were thermally processed and hot filled rendering them shelf-stable. The refrigerated ready-to-eat (RTE) category contained a variety of products and ingredients, but all were perishable. While the ingredients used in formulation of the refrigerated RTE foods were either thermally processed or microbiologically inert, through combination and handling during production, the final product was not stable outside of temperature control.

"Spoilage" was determined by the manufacturer as a quality deviation in the product specifications that rendered the food unacceptable for consumption. The spoilage characteristics in products received from manufacturers included visible mycelial development, turbidity in liquid products, gas production, and organoleptic fault. Manufacturers submitted deviated product to the extension programs for microbiological examination, as described below. Once received, products were stored under refrigeration at 4°C for up to two days until processed.

Identification of intrinsic and extrinsic microbial controls

For each of the spoiled products, data was collected on the physicochemical properties and associated processing conditions. Critical factors used in processing were recorded to establish the type and degree of thermal or non-thermal microbial reduction step. Additionally, a single temperature value was assigned to represent the thermal treatment for each product. For products which were cold filled, the temperature was recorded as 25°C. The fill temperature was used for hot filled products. The baking temperature was used only if the water activity of the product was reduced below 0.61 during baking, otherwise it was treated as a "cold filled" product due to the potential for contamination and growth of spoilage fungi following the thermal process. Deviations to the scheduled process, if known, were recorded. Intrinsic microbial controls associated with product formulation were also determined. The final pH of each product was determined using a pH meter (Accumet Basic AB15, Fischer Scientific, Pittsburgh, PA) and the water activity was determined using a water activity meter (AquaLab 4TE water activity meter, Decagon Devices Inc., Pullman, WA). The oxygen availability in the container was assessed by sorting each product into one of two categories: high oxygen tension or reduced oxygen tension based on the packaging conditions. Reduced oxygen tension products included vacuum sealed, hot filled, high acid foods; cold filled, hermetically sealed, fermented foods; and vacuum packaged products. When an unopened container of product was provided by the manufacturer, vacuum strength (inches Hg) was measured using a Canners Vacuum Gauge with a rubber collar (10816-00 vacuum gauge, Wilkens-Anderson, Chicago, IL). The continuous predictor variables pH and a_w were transformed into categorical variables for some of the data

visualizations and analyses described below. Low pH and high pH categories were delimited based on the regulatory cutoff for inhibition of *Clostridium botulinum* germination at pH 4.6. Water activity levels of low (<0.6-0.85), intermediate (0.85-0.95), and high (>0.95) based on cutoffs specified in Sperber and Doyle (2008) which are closely related to those used in food safety regulations, a_w <0.93 inhibits *Clostridium botulinum* germination, and a_w <0.85 inhibits *Staphylococcus aureus* toxin production, the lowest water activity that supports the growth of foodborne bacterial pathogens. Although these categorizations are framed largely around the growth potential of bacterial pathogens, these cutoffs are, for this reason, often targeted as critical limits by food processors. Therefore, grouping spoilage according to the convention utilized by manufacturers is most likely to result in usable information.

Isolation of fungi from spoiled product

Isolation of fungi from solid foods was performed by removing contaminated pieces of spoiled product and placing mycelia sections near the center of a Petri dish containing unacidified Potato Dextrose Agar (Becton, Dickinson and Co., Franklin Lakes, NJ). Although a range of growth media for culturing fungi from various environments exist, PDA was selected as an all-purpose growth substrate (Sperber and Doyle, 2009). Additionally, the collection is overrepresented with spoiled juice/acidic beverages for which PDA is well suited for culturing the causative fungus. For products with lower water activity, which may have selected for spoilage organisms less well suited for growth on PDA, confirmation of recovery was supported by mycelial development on spoiled products so recovery could be visually confirmed. For liquid products and those without visible mycelial development (e.g. yeast spoilage), a product

sample (10 g) was serially diluted in 0.1% peptone water (Becton, Dickinson and Co., Franklin Lakes, NJ) and plated on PDA. Depending on the product, low levels of various background biota appeared on the lower dilutions. However, the predominant spoilage microorganism could be identified at the 10^{-3} dilution or greater. Plates were incubated at 25°C for seven days, and isolates were then subcultured and incubated for up to 30 days to facilitate spore development. Freezer stock was prepared for long-term storage (-80°C) in 20% glycerol. Rarely (6%), bacteria were identified as the causative spoilage agents. In the case of *Alicyclobacillus* spp. (n1 in the collection), colonies appeared on PDA and the identity was confirmed through sequencing of the 16S region. In all other cases (7/127), when no fungi could be isolated from the product, additional testing on Trypticase Soy Agar (Becton, Dickinson and Co., Franklin Lakes, NJ) lead to the identification of the bacterial spoilage organism. By virtue of the processing and storage conditions, pH, and water activity of the products involved in this study, the vast majority of the spoilage agents were yeast and filamentous fungi.

Identification of fungal isolates

Genus-level identification was made by sequencing the ITS barcode region. Isolates were grown on PDA and DNA was extracted from scraped colonies using the PowerSoil DNA isolation kit and following the manufacturer's instructions (Qiagen, Hilden, Germany). The internal transcribed spacer region, ITS 1 and 2 which flank the 5.8S gene, was amplified using the Primers ITS4 (5'TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') as described by White (1990). PCR products were confirmed by gel electrophoresis, purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced by the Cornell

University Life Science Core Laboratories Center (Ithaca, NY) using a ABI PRISM 3730 DBA sequencer (Applied Biosystems, Foster City, CA). The resulting sequence was compared to the NCBI BLAST nucleotide database, excluding uncultured microbes. Any bacterial spoilage was only identified as "Bacteria" within the data set.

Food spoilage-relevant isolates, *Eurotium* (*Aspergillus*) and *Neosartorya* (*Aspergillus*) were so noted as they are functionally distinct from other *Aspergilli* based on their capacity to survive heat processing (*Neosartorya* (*Aspergillus*)) and growth potential in low water activity environments (*Eurotium* (*Aspergillus*)). The teleomorphic names are still recognized and used by the food industry, therefore they were retained for clarification in this study. Assignments were based on (Samson et al., 2009 and Samson et al., 2007). Similarly, the heat resistant mold *Paecilomyces/Byssochlamys* was referred to as such within the publication. Again, the name associated with the sexual state, *Byssochlamys,* is widely recognized within the food industry. In each of these cases, the name of the food-spoilage relevant form, commonly used within the industry, was applied in addition to the holomorphic genus name. During the analysis, the distantly related Mucorale isolates (*Mucor, Rhizopus,* and *Syncephalastrum*) were grouped together.

Root cause analysis of select quality failures

Several of the spoiled products (n11) were part of short and long-term studies (e.g. Snyder et al., 2016; Daniels et al., 2017) to determine the environmental source, process failure, or etiological agents associated with notable quality deviations. The full descriptions of spoilage issues for which a root cause was determined are included in Table 1. For root cause analyses that required the differentiation between post-process

contaminants and heat resistant filamentous fungi, cursory tests were implemented to assess heat resistance as adapted from (Samson, 2010). Briefly, 30-day old plates were scraped with phosphate buffered saline (pH 7.2) and equal volumes were transferred to a thin-walled plastic bag (Whirl-Pak, Uline, Pleasant Prairie, WI) and heat sealed. Bags were submerged in a hot water bath at 70°C for up to five minutes, and a single bag was pulled every minute. Heat-treated samples were serially diluted and spread plated on PDA for enumeration of survivors. Although this method does not definitively prove that the spoilage isolate would survive the processing conditions relevant to the product from which it was isolated, it does allow for rapid identification of post-processing contamination for isolates which could not possibly survive those conditions with a minimal investment of resources.

For confirmation that a specific isolate was the cause of a given spoilage defect, Koch's postulates were applied (Sperber, 2009). The putative spoilage agent must have been cultured from the spoiled product, and grown in pure culture for identification as described above. The putative spoilage isolates were then reintroduced into fresh product and spoilage characteristics were observed before re-isolation of the spoilage agent. Introduction of the putative spoilage agent was made under conditions specific to the relevant point of production.

Statistical analysis

Statistical analysis was performed using R statistical software (version 3.0.1, R-project, Vienna, Austria). The bipartite network analysis was developed using the R package ggbipart (Fig. 2). A classification tree (Fig. 4) was constructed using the rpart package. Partitions were made based on the predictor variables of product category,

process regime, pH, and water activity for genera isolated with at least four unique observations. This reduced data set contained 117 observations. The yeast genera remaining were collectively assigned to a single categorical outcome "yeast" which reduced the overall number genera in the model to ten. At least 20 observations were required to create a subsequent split to minimize over fitting. At terminal nodes, additional outcomes were added for all genera with a probability of 0.15 or greater. An external validation set was generated unique from the training set through blinded observations (20) were gathered from the published literature. The validation error was determined to be 0.6 for the presented model. Notably, none of the observations from the literature included heat sensitive mold (i.e. Penicillia) in hot filled product.

3.3 Results

Diversity of fungal spoilage isolates

Only spoiled products were analyzed, the total background biota in unspoiled product was not assessed as in Garnier et al., 2016; Al-Bulushi et al., 2017, and De Clercq et al., 2015. As a consequence, only one isolate was identified from each spoiled product. Spoilage organisms (127) were isolated from commercially manufactured products, of which only 9 were bacteria, while 26 were yeast and 92 were filamentous fungi. All of the isolated fungi belonged to the Ascomycete phylum, except for four Mucoralian isolates from the genera *Mucor, Rhizopus,* and *Syncephalastrum* which were the most distantly related fungi isolated in this study. Eight unique yeast genera were identified and included *Rhodotorula, Pichia, Candida, Torulapsora, Sacharomycopsis, Aureobasidium, Saccharomyces,* and *Exophiala*. The vast majority of

these isolates belonged to the family Saccharomycetaceae, in addition to one isolate from each of Aureobasidiaceae and Herpotrichiellaceae. Fourteen genera of ascomycete filamentous fungi were identified from two major groups, Eurotiomycetes and Dothideomycetes. Of the 92 filamentous fungi, 42% were from the Trichocomaceae family. Some genera were only isolated once and these included *Monascus*, *Epicoccum*, *Talaromyces,* and *Sarocladium*. Other genera were isolated repeatedly. The occurrence of filamentous fungi which appeared multiple times ranged from 2% (*Phoma*) to 18% (*Penicillium*). Around half (57%) of isolates came from low pH (< 4.6), and high water activity (65%) products (Fig. 1) which are common physicochemical conditions in foods. The majority of isolates were obtained from high water activity (>0.95) products due to both the abundance of juices within the data set and to the increased availability of water to support fungal growth. Products were manufactured from fruits and vegetables (49%) and included, juices, preserves, sauce/relish, and fermented hot sauces; dairy (5%) and included yogurt, cheesecake, and cheese; and nuts and grains (16%) and included granola, cookies, cake, nuts, seeds, sunflower oil, and tempeh.

The most frequently occurring genus was *Penicillium* (n23) which was isolated from all processing conditions and all product categories except fruit preserves - although this may be due to the low number of observations for this category since Penicillia has been reported to cause spoilage of these products (Thompson, 2009). *Penicillium* is one of the most prevalent spoilage molds. (Sharpe and Pettipher, 1983). Of the 23 *Penicillium* isolates identified, 10 were post-processing contaminants in hot fill products while the remaining 13 isolates were from pasteurized or non-thermally processed products, which indicates the diversity of conditions under which Penicillia

were capable of causing spoilage. Yeast were isolated from all product types except fruit preserves, tomato-based sauces, and dried cereals and nuts, and from all process conditions except hot fill. This is aligned with reports of yeast heat sensitivity and association with high water activity environments. Although yeast can spoil concentrated sugar solutions (confection products), they are less common in dry or high salt solutions (Wang et al., 2015). Yeast are responsible for 75% of acidified food spoilage (Sharpe and Pettipher, 1983) but are heat sensitive. Subsequently they were readily isolated from pasteurized juice (11 of the 26 yeast isolates were from spoiled pasteurized juices), but not from hot filled, shelf stable juices. *Candida* was the most frequently isolated yeast genus followed by *Rhodotorula*, *Pichia*, and *Torulaspora*. Rarely, yeast were isolated from baked goods and oils (n3). Because of the reduced water activity, baked goods are more frequently spoiled by filamentous fungi (71%) but some cases of spoilage by yeast and bacteria have been reported (Sperber and Doyle, 2009). *Sacharomycopsis* has been isolated from oils used as processing aids in bread production accounting for over half of the spoilage isolates present in blade oil (Legan and Voysey, 1990) and in this study, may have resulted from condensation in container while product was warm.

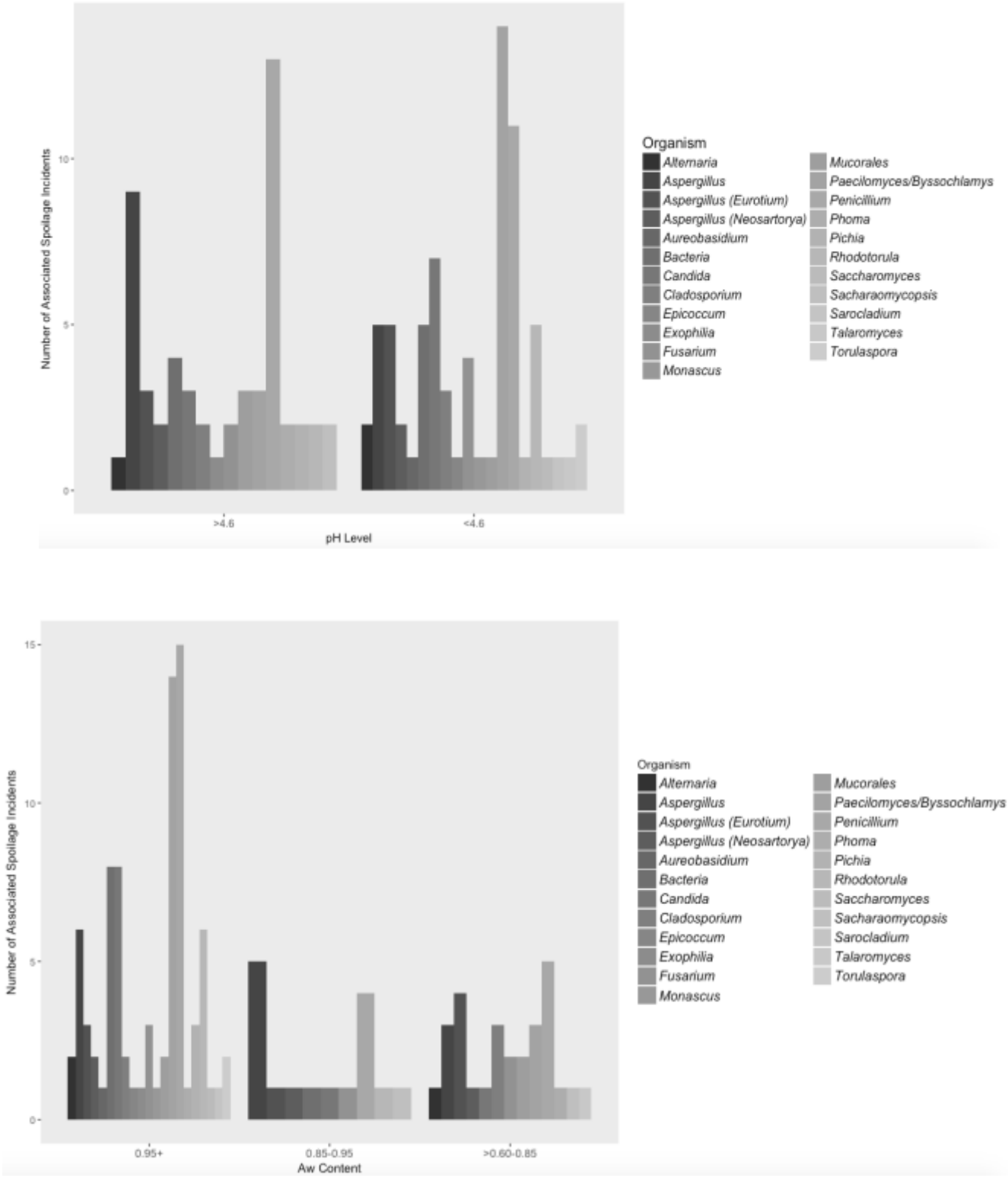

Figure 3.1: Distribution of spoilage fungi by genus in products of high and low pH (above) and high, intermediate, and low water activity (below).

Association of fungal genera with process regimes and product category

Fungal spoilage genera are associated with process regime (Fig. 2) and product category (Fig. 3). Although these predictors are sometimes related (e.g. fruit preserves were always hot filled), there were many cases when they diverged (e.g. juice/acidic beverages were either hot filled or pasteurized), which impacted the identity of associated spoilage fungi. The network analysis shown in Fig. 2 illustrates the number of observations for each identified fungal genera under given processing conditions. Pasteurization was least selective and 15 different genera were capable of spoiling products manufactured under this processing regime. This process category was almost entirely composed of juices/acidic beverages were refrigerated to prevent spoilage.

Hot filled products (juices, fruit preserves, tomato-based sauces, confections) were spoiled by HRM (*Paecilomyces/Byssochlamys, Aspergillus* (*Eurotium*)*, Talaromyces,* and *Aspergillus*(*Neosartorya*)) in addition to other filamentous fungi (*Penicillium, Monascus, Fusarium*) which indicated that these products were susceptible to contamination of ingredients with HRM as well as spoilage from post-processing contamination or under-processing. In fact, only about half of the isolates obtained from hot fill products were HRM, which is likely an over estimation of the isolation frequency for these organisms generally in commercially spoiled products. This suggests that environmental contamination is likely to be a cause of spoilage despite manufacturers' frequent concerns to the contrary (Table 1). The most frequently isolated HRM was *Paecilomyces/Byssochlamys* with 16 observations. Ingredients are the most common source of *Paecilomyces/Byssochlamys* and 1-6% of susceptible raw ingredients are reportedly contaminated (Rico, 2016). Post-processing contamination of

HRM is less likely to result in spoilage since the thermal process is often essential in initiating germination of the ascospores. Yeast were associated with pasteurized, fermented, and non-thermally processed products but not hot fill, since no spoilage yeast relevant to the food industry is known to produce highly heat resistant ascospores. Baked products were infrequently spoiled by yeast since the minimum water activity required to support yeast growth is often greater than that in many baked products. HRM were almost exclusively isolated from hot filled products, with the exception of a single *Paecilomyces/Byssochlamys* isolated from spoiled cheesecake. Houbraken et al. (2008) reported the detection of a single *Paecilomyces/Byssochlamys* isolate from spoiled rye bread in a collection of 16 total isolates; however, that isolate lacked significant heat resistance compared to the rest of the collection.

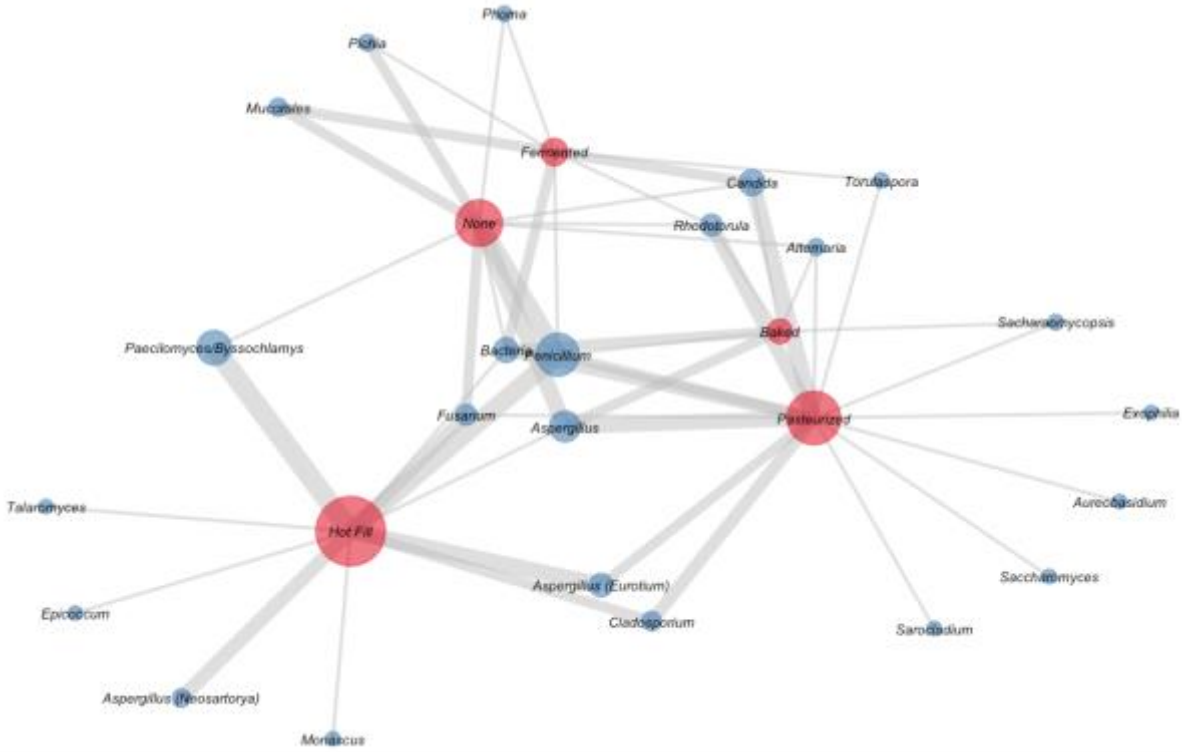

Figure 3.2: Network analysis of specific spoilage organisms (blue) and processing regime (red). Radius represents number of observations of the organism or process. Line thickness represents number of spoilage incidents.

The physicochemical properties and ingredient-associated biota for product categories also select for specific spoilage genera. For example, eight *Aspergillus* (*Eurotium*) isolates were identified in this collection and were taken from products with an average water activity level of 0.82. This is in keeping with previous work by Pitt and Hocking (1977) on this xerophillic fungus. The *Mucorales* (*Mucor, Rhizopus,* and *Syncephalastrum*), although prolific spoilers of raw fruit and vegetables, were infrequently isolated from processed products as they are highly sensitive to heat (Snyder et al. 2016) and favor raw agriculture commodities (Pitt and Hocking, 2009). However, Mucorales were isolated four times in this study from fermented dairy products and dried nuts. Spoilage of fermented dairy products by *Mucor* and *Rhizopus* has previously been reported, as has spoilage of dried seeds by *Rhizopus* and *Syncephalastrum* (Adebajo et al., 1994). Yeasts are the major cause of spoilage for yogurt because the low pH provides a selective environment and companies often employ a quality standard of <10 CFU/g yeast and mold for finished product micro testing, while >100 CFU/g has been associated with a decreased shelf life (Sperber and Doyle, 2009). *Candida, Torulaspora,* and *Mucor* (yeast-like phase) were isolated from yogurt within this study and were associated with sanitation issues (Table 1). Mucorales, yeast, and bacteria were associated with refrigerated RTE and fermented products (Fig. 3), while the Trichocomaceae tended generally towards the juices, fruit preserves, and tomato-based sauce side. However, *Penicillium* and *Aspergillus* were found across the spectrum of product categories tested. The specific spoilage organism associated with juice, by far the largest product category in this study, depended largely

on the processing conditions.

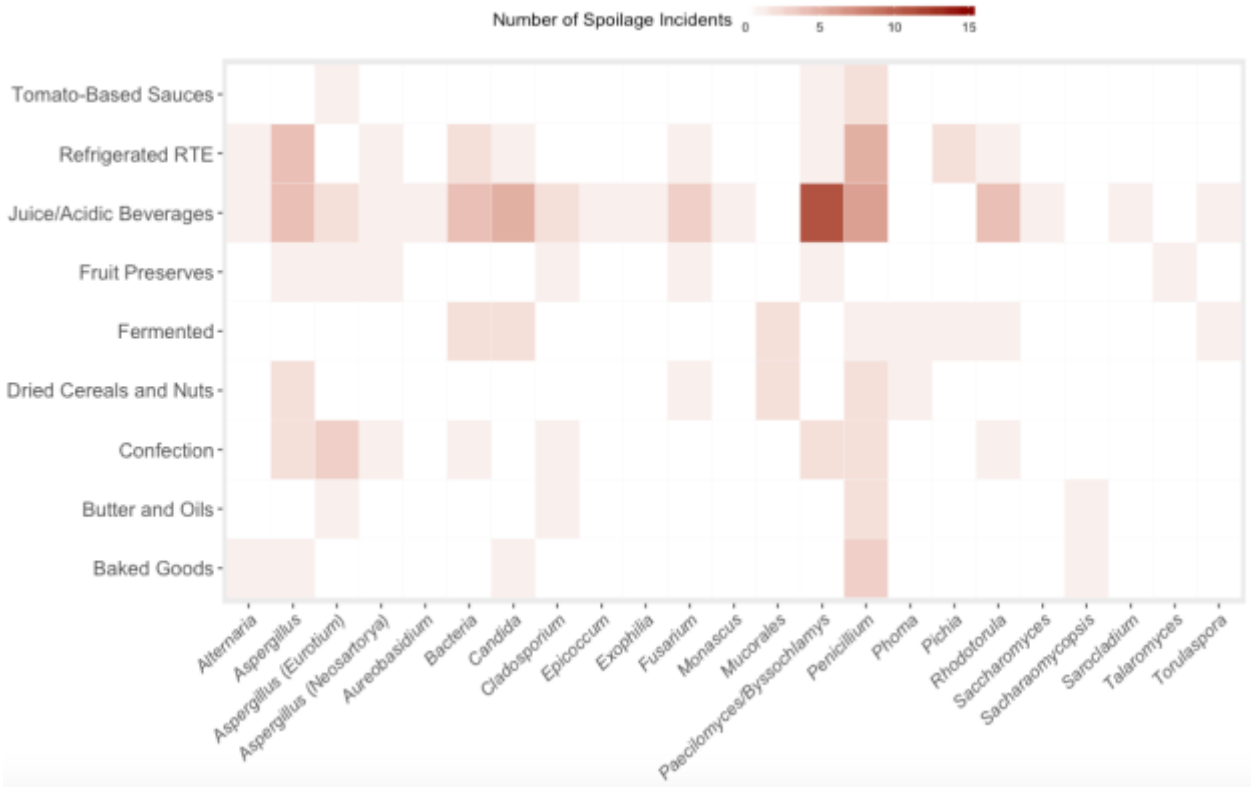

Figure 3.3: Stratification of fungal genus isolated from commercially spoiled products by product category. Shaded cells represent increasing number of observations. White cells represent organism and process combinations with zero observations.

Physicochemical properties predictive of dominant spoilage genera

The probability of isolating particular genera from a given product based on physicochemical attributes and processing conditions are presented in Fig. 4. These predictions were generated by recursively partitioning observations for the ten genera which had at least four observations. The first split in the classification tree is based on processing conditions, hot fill products are directed to the right of the tree while other processing conditions are directed to the left. The hot fill branch was enriched in HRM (*Paecilomyces/Byssochlamys, Aspergillus* (*Eurotium*), *Aspergillus* (*Neosartorya*)) while some heat sensitive fungi, like yeast, were excluded. However, other environmental

contaminants, *Cladosporium* and *Penicillium,* were predicted in hot fill products at 0.15 and 0.17, respectively, among other observed heat sensitive molds at lower frequencies not included in this figure. This suggests the propensity for failures in production to result in microbial spoilage of a product accounts for a sizable portion of the spoilage incidents. The probability of isolating *Paecilomyces/Byssochlamys* was greatest (0.57) in high water activity, hot filled products likely due to the proportion of the juice/acidic beverages which were spoiled by this organism. Moreover, *Paecilomyces/Byssochlamys* is reportedly inhibited by water activity limitation. In contrast, *Aspergillus* (*Eurotium*) had the highest probability of isolation from low water activity, hot filled products (0.23) and is known for low water activity tolerance.

The left side of the tree, products that were pasteurized, fermented, baked, or non-thermally processed, were further broken down by product type. Confections, pasteurized juices, and fermented products were split by pH. Products with a near neutral pH (>6.7) which included vegetable juices, cheese, and chocolate products were most likely to be spoiled by bacteria (0.43), *Aspergillus* (0.29), and *Cladosporium* (0.14). The remaining products, refrigerated RTE, cereals and nuts, baked goods, and butter and oil were spoiled by *Aspergillus, Penicillium,* yeast, and with a lower propensity, Mucorales and *Fusarium* depending on pH. In several instances, these products should not have spoiled by these organisms by virtue of process or water activity. However, production and sanitation failures resulted in spoilage by opportunistic fungi so that some of the high probability spoilage fungi identified in the classification tree were associated with production failures specific to the product as much as thermal process or physicochemical properties.

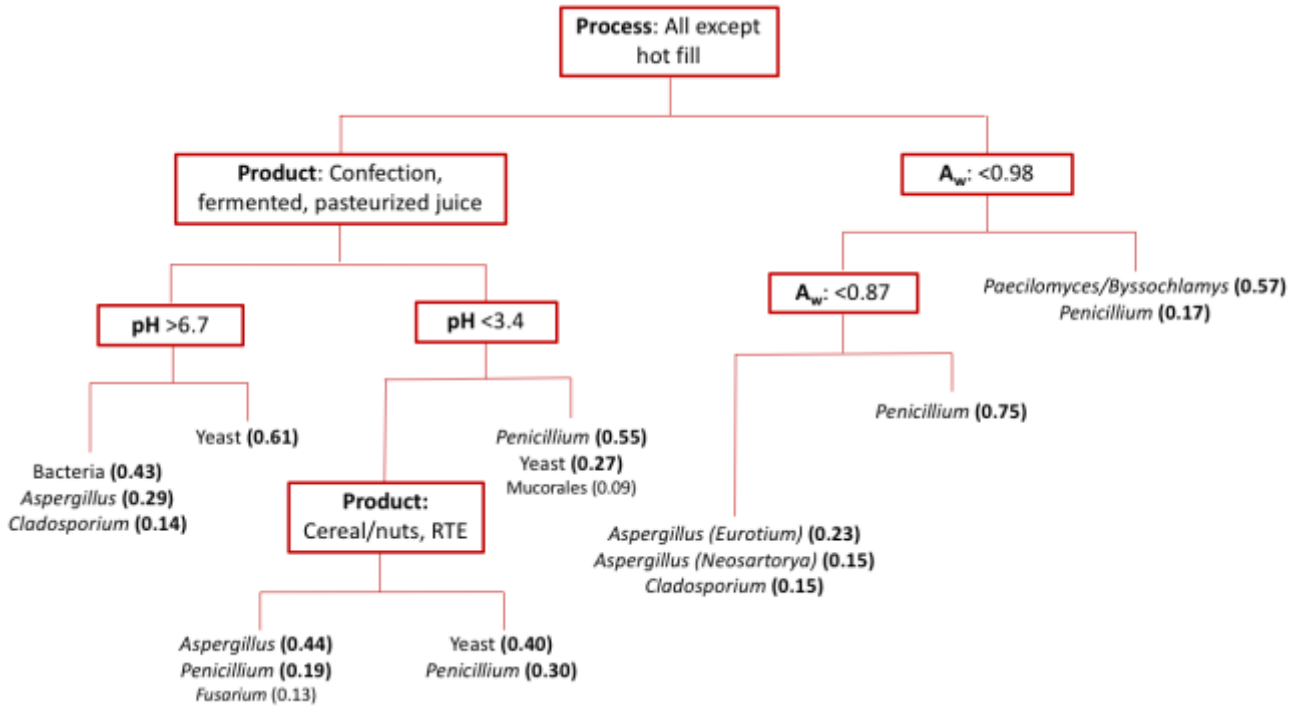

Figure 3.4: Classification tree dividing fungal spoilage genera based on processing conditions and physicochemical properties of the product. The criterion at each split partitions spoilage fungi into homogenous subsets, left-handed branches are enriched in observations that align with the node criterion. Probabilities for each genus at a leaf position are included parenthetically.

Identification of production failures contributing to spoilage incidents

The product associated attributes of physicochemical properties, processing regime, and product category influence the specific spoilage organism. However, the identity of the spoilage organism is not exclusively predictable based on these features (Fig. 4). Production failures likewise influenced the spoilage outcome. As discussed above, the probability of hot filled products spoiling due to HRM compared to post-processing contamination is an indication of the mediating influence of production failures, or inaccurate expectations that GMPs and SSOPs are either infallible or of little consequence. Root cause was identified for several of the spoilage incidents included in this study. A summary of the most common problems that lead to spoilage, and specific

product and organism examples of these incidents, are presented in Table 1. In several of the cases, the product was under processed through reduced thermal treatment or because of inadequate acidification. The pH and type of organic acid influence the accumulated lethality during thermal processing, therefore elevated pH levels increase the probability of survival for contaminating fungi (Sastry, 1986). In these cases, filamentous fungi with modestly heat resistant hyphae/asexual spores were identified as the associated spoilage organism. These Penicillia and *Monascus* isolates did not meet the conventional definition for HRM, they were inactivated by a thermal treatment of 20 min at 80°C (Samson, 2010). However, a moderate heat resistance contributed to the spoilage potential for these organisms, and so the industry may consider the adoption of an additional classification for spoilage fungi capable of spoiling heat treated products when a process deviation has occurred. *Monascus* has previously been associated with moderate heat resistance (Panagou et al., 2002) as have some resistant Penicillia hyphae (Thompson, 2009).

Other elements influencing spoilage fungi that are not captured in physicochemical product properties and processing conditions included cleaning method, packaging type and method, and equipment design. Inclusion of these factors in predictions of pertinent spoilage fungi may improve quality risk assessments. In dry environments, such as those used in the production of baked goods, fats and oils, and confections, the dry-cleaning regimen used by manufacturers is relatively ineffective against filamentous fungi (Rico, 2016) which may increase the potential for accumulation of fungal spores. Packaging was also identified as a production step and a source of spoilage fungi. Since packaging serves as the barrier against the ingress of spoilage organisms throughout

shelf-life, the integrity of that barrier and sanitary transfer of the product to the package are crucial steps in quality protection. In instances of low water activity product spoilage, we identified low vacuum strength and the potential for elevated oxygen levels in container headspace as contributing to product spoilage, along with the potential for condensation development in water activity controlled foods (Table 1). Thermal process also intersects with total package oxygen as fill temperature directly correlates with vacuum strength as well as dissolved oxygen (Sperber, 2009). The fill step is highly susceptible to post-processing contamination. Preventing spills, condensation, and correctly forming package seams are requisites for controlling spoilage and are usually addressed through GMPs. However, the results of this study suggest that failures at this step are not infrequently the cause of spoilage incidents. Sanitation and sanitary design is another major category of production failures contributing to spoilage. Equipment design and maintenance as well as environmental sanitation influences the possibility of post-processing sanitation. Effective preventative maintenance programs and verification of these sanitation systems may be useful in production environments which rely on these programs for quality control.

Table 3.1: Identification of process failures and associated spoilage incidents

Problem	Product and Organism	Commercial Spoilage Example	Analysis
Problematic Raw Ingredients	Apple juice concentrate, *Alicyclobacillus* and *Paecilomyces/Byssochlamys*	Contaminated fruit used to produce concentrate became problematic when diluted back to single strength juice.	High volume detection methods; verification of the COA
	Cheese, *Mucor*	Contaminated fungal-synthesized enzyme used in production of the fresh cheese lead to hyphal growth.	Isolation of *Mucor* from enzyme stock and also spoiled product
	Cherry juice, *Fusarium*	Repeated detection of the same isolate from product made using the same fruit supplier.	Tracking trends in microbiological testing results
Post-Processing Contamination	Tomato sauce, *Penicillium*	Mycelial development environmental contamination during the fill step.	Cursory check of heat tolerance from isolated fungus
	Yogurt, *Mucor*	Product contamination due to poor sanitary design followed by temperature abuse.	Challenge studies under various production scenarios
	Yogurt, *Candida*	Similarly, product contamination due to ingress of yeast from the environment into the fermentation vessel.	Identification of GMPs violations and sanitary design short comings
Under Processing	Shelf stable fruit juice, *Penicillium*	Pellicle development in the headspace occurred because the minimum fill temperature specified in the scheduled process was not achieved.	Review of production records from toll processor and lack of appropriate corrective action
	Pumpkin butter, *Aspergillus (Eurotium)*	Maximum pH specified in the scheduled process was exceeded, resulting in a decreased cumulative lethality.	Review of production records and lack of appropriate corrective actions
	Granola, *Penicillium*	Water activity of the final product was too high to maintain shelf-stability.	Comparison of a_w to reported growth requirements
Packaging Failures	Raspberry jam, *Paecilomyces/Byssochlamys*	A sufficient vacuum was not achieved during production which elevated total package oxygen.	Vacuum strength assessment using a canner's vacuum gauge
	Vegetable oil, *Aspergillus*	Condensation due to temperature fluctuations created a local environment with elevated a_w.	Destructive product evaluations and review of production conditions
	Caramel sauce, *Cladosporium*	Product spill around package seal created conduit for environmental contaminants.	Evaluation of container integrity

3.4 Conclusions

Spoiled products collected through commercial submissions to our food microbiology extension programs revealed associations between the identified spoilage fungi and aspects of the formulation and processing of the product. Generally, only a few genera of spoilage fungi were likely to contaminate a given product based on the probability of exposure (on ingredients, in the production environment) and survival and growth within the product. Moreover, only a single fungus was identified as the predominant spoilage organism in each spoiled product, although several units of the same product may have been spoiled by different genera, particularly if the cause of spoilage was post-processing contamination.

For manufacturers, the identification of the target spoilage organisms and selection of control strategies should be risk based, and knowledge of the pertinent spoilage organisms and dictating parameters may help optimize food quality control strategies including preservative profiles, hygienic measures, and identification of critical quality points. In this study, fungi were isolated and identified from commercially spoiled products rather than the identification of all fungi present in the environment or detectable in the finished product, which is useful in establishing specific spoilage organisms and predictive processing conditions. However, the association of these environmental fungi with spoilage potential and identified spoilage organisms is essential in determining indicators and targets for environmental monitoring (Filtenborg et al., 1996). In addition to the "intrinsic" and "extrinsic" factors of pH, water activity, processing conditions, and product type previously identified in the literature as

predictive of spoilage organism, mediating factors such as packaging system, the propensity for post-processing contamination, and the sanitation system were also identified as strong predictors of specific spoilage organisms. This suggests that quality plans should consider the most likely production failures specific to their system. Sharpe and Pettipher (1983) suggested using "defect rate" as opposed to shelf-life as a quality metric which would be more useful for tracking trends in sporadic contamination and determining problem areas during production, particularly among the extended shelf-life products examined in this study. In contrast, shelf-life monitoring is useful for perishable products subject to high variability in microbial quality for raw ingredients. The use of statistical process control or quantitative microbial risk assessments may be useful in order to address the complexity of risk-based spoilage predictions and the increased importance of spoilage prevention in order to capture the stochastic and multidimensional aspects of controlling microbial spoilage.

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CHAPTER 4

Characterization and control of *Mucor circinelloides* spoilage in yogurt

ABSTRACT

Consumer confidence in the food industry is severely affected by large-scale spoilage incidents. However, relatively little research exists on members of the fungal subphylum Mucormycotina (e.g. *Mucor*), which includes dimorphic spoilage organisms that switch between a yeast-like and hyphal phase depending on environmental conditions. The presence alone of *Mucor circinelloides* in yogurt will not cause spoilage, but outgrowth and subsequent changes in quality (e.g. container bloating) can cause spoilage if not controlled. The purpose of this study was to evaluate the effects of pasteurization regime, natamycin concentrations, and storage temperature in yogurt production on *M. circinelloides,* as measured by fungal proliferation and carbon dioxide production.

M. circinelloides was isolated from commercially spoiled yogurt. Compared to clinical isolates and industrial strains of *M. circinelloides,* the spoilage isolate showed increased yogurt-spoilage potential. *D*-values and *z*-values were determined for the spoilage isolate in milk as an evaluation of the fungus' ability to survive pasteurization. Natamycin was added to yogurt at 0, 5, 10, 15, and 20 ppm (μg/ml) to determine its ability to inhibit *M. circinelloides* over the course of month-long challenge studies at 4°C, 15°C, and 25°C. Survivors were recovered on acidified PDA and carbon dioxide levels were recorded. The *D*-values at 54°C, 56°C, and 58°C for hyphae/asexual spores were (in min) 38.31 ±0.02, 10.17 ±0.28, and 1.94 ±0.53, respectively, which yielded a

z-value of 3.09°C. The *D*-values at 51°C, 53°C, and 55°C for yeast-like cells were (in min) 14.25 ±0.12, 6.87 ±1.19, and 2.44 ±0.35, respectively, which yielded a *z*-value of 0.34°C. These results indicated that *M. circinelloides* would not survive fluid milk pasteurization if contamination occurred prior to thermal treatment. CO_2 production was only observed when *M. circinelloides* was incubated under low-oxygen conditions, and occurred only at temperatures above 4°C. Addition of 10 ppm and greater of natamycin inhibited the growth and CO_2 production of *M. circinelloides* under moderate temperature abuse when compared to the untreated control.

These data suggest that yogurt spoilage (container bloating) caused by anaerobic growth of *M. circinelloides* is due to post-pasteurization contamination. Temperature abuse facilitated spoilages as CO_2 production was observed in yogurt incubated at 15°C and 25°C, but not at 4°C. The addition of at least 10 ppm of natamycin was found to be effective at preventing *M. cicinelloides* growth in both hyphal and yeast-like phases, as well as CO_2 production in temperatures of up to 15°C for 30 days.

4.1 INTRODUCTION

Microbial spoilage of food increases waste, reduces consumer confidence in the food industry, and decreases food system sustainability. In the fall of 2013, a large spoilage incident resulted in a national recall of Greek yogurt contaminated with the fungus *Mucor circinelloides*. In total, about a month worth of production was recalled for potential contamination, and the event received national media attention. The company initially implemented a product withdrawal, but after several hundred consumers reported symptoms of gastroenteritis, the withdrawal was changed to a voluntary recall

(USFDA 2015). A subsequent paper analyzing the spoilage organism's potential for pathogenicity found that in a murine tail vein injection model, the *M. circinelloides* f. *circinelloides* spoilage isolate was virulent. However, mice that ingested the spoilage fungus, instead of being injected with it, experienced no significant ill effects, although the fungus was shown to survive the murine gastrointestinal tract (Lee et al. 2014). *M. circinelloides* is recognized as an opportunistic pathogen, principally impacting people recovering from invasive medical procedures (Schwarz et al. 2006). In the context of food and agriculture, *Mucor* is a recognized secondary pathogen of plants, causing produce spoilage (Jones and Aldwinckle, 1990), but not generally considered a foodborne pathogen because ingestion of the fungus has not been linked with gastrointestinal illnesses, especially among healthy individuals. Although, there is a 2014 report in the Deleware Medical Journal of a single case of rhinocerebral infection of *M. circinelloides* in an immunocompromised individual who had consumed the spoiled product (Lazar et al. 2014) which is emblematic of the opportunistic behavior of this organism. The subphylum of basal fungi, Mucormycotina, includes *Mucor* and *Rhizopus*, which are identified in the field by their white, aerial hypha frequently found on fruits including apples, pears, and strawberries (Alexopoulos et al. 1996; Pitt and Hocking, 2009). *Mucor* and *Rhizopus* have also been used in the production of various fermented foods including tempeh and sufu pehtze (Han et al. 2004). A recent study suggested *M. racemosus* extracts have an anti-inflammatory effect, which was proposed for use as a potential treatment option for chronic inflammation (Meier et al. 2015). Taken together, *Mucor* spp. can be opportunistic pathogens, desirable fermentative organisms, or perpetrators of food spoilage, depending on the context and

characteristics of individual isolates.

When compared to foodborne pathogens, food spoilage organisms have received relatively little attention. Food safety dictates the vast majority of regulatory policy and the majority of research grants available are likewise geared toward advancing food safety. However, food quality has major implications for sustainability and world hunger (FAO 2012). Additionally, food spoilage incidents can significantly impact food companies as consumers' confidence in the food industry is shaken by product withdrawals and the experience of purchasing a product that spoils before the end of shelf-life. Spoilage of the *M. circinelloides*-contaminated cups of Greek yogurt was characterized by bloating and bubbling due to CO_2 production by the fungus.

M. circinelloides is a dimorphic fungus and has two distinct growth phases, yeast-like and hyphal phase, and will switch between the two growth phases depending on environmental conditions (Orlowski 1991). The exact conditions that trigger yeast-like growth are strain dependent, but are typically characterized by some combination of low environmental oxygen, high carbon dioxide levels, and/or reduced hexose availability (Wolff et al., 2002). Food manufacturers generally consider molds to be obligate aerobes, and use anaerobic or reduced oxygen conditions to control for mold spoilage (Norholt and Bullerman 1982; Ray and Bhunia 2008). The dimorphic growth of Mucormycotina proves an exception to that rule. In fact, CO_2 production, which lead to the yogurt cup bloating and fizzing observed by consumers, specifically occurred during yeast-like growth in low-oxygen conditions.

Foschino et al. (1993) report spoilage of commercial yogurt in several brands due to fungal contamination, including *Mucor* spp. As in the spoilage incident discussed here

which occurred >20 years later, the authors report inoculation of these isolates into yogurt cups resulted in container bloating and elevated CO_2 levels in the container headspace. Because relatively little and intermittent research exists characterizing the spoilage of dairy products by *M. circinelloides,* a systematic determination of the conditions which facilitate spoilage, and those which control it, would aid in the prevention of major spoilage events in the future. The objective of this study was to evaluate the effects of pasteurization regime, natamycin concentrations, and storage temperature on *M. circinelloides.*

4.2 Materials and methods

Microbial cultures

The recall-associated *M. circinelloides* strain isolated from spoiled yogurt and two clinical isolates were generously provided by the Joseph Heitman at Duke University (Durham, NC). The *M. circinelloides* strains 3615, 3614, and 3627 were obtained from USDA's Agricultural Research Service NRRL collection (Table 1). All isolates were maintained as frozen stock cultures and plated on Potato Dextrose Agar (Becton, Dickinson and Co., Franklin Lakes, NJ) acidified to pH 3.5 prior to use in experiments. Inoculum was prepared by scraping plates which had been incubated at 25°C for 48 h and re-suspending the hyphae/asexual spores in peptone water (Becton, Dickinson and Co., Franklin Lakes, NJ) or skim milk, depending on the experiment.

Yeast-like cells used to generate thermal destruction data were prepared by streaking *M. circinelloides* onto acidified PDA. Plates were incubated at 25°C for at least 48 h in a GasPack anaerobic jar (Becton, Dickinson and Co., Franklin Lakes, NJ).

Typical colony and cellular morphology was observed following incubation. Following extraction from GasPack anaerobic jars, yeast-like cells were immediately used thermal destruction experiments.

Inoculation of yogurt cups

The time required for various *M. circinelloides* isolates to produce sufficient CO_2 to visibly spoil yogurt cups was determined by inoculating single serving yogurt cups (commercial retailor, Geneva, NY) with 10^4 CFU/g yogurt of asexual spores from a given *M. circinelloides* isolate. Adhesive rubber septa (Thermo Fisher Scientific Inc., Indianapolis, IN) were fixed to the foil top and inoculum was delivered via a sterile needle. Cups were incubated at 25°C until CO_2 production resulted in container swelling which was visibly detectable.

Hyphal phase product inoculation

In all experiments reporting hyphal phase growth, commercially available yogurt was purchased from a retailor (Geneva, NY) in 850.50 g (32 oz) units and 30 g of yogurt was transferred to individual stomacher bags. Yogurt was inoculated with *M. circinelloides* (approximately 10^4 CFU/g yogurt), homogenized, and transferred by weight into sterile stomacher bags. Yogurt samples were stored aerobically at designated storage temperatures (4°C, 15°C, and 25°C) for the challenge study. All data comparing growth rates of various *M. circinelloides* isolates (Figure 1) were collected from yogurt incubated at 25°C.

Yeast phase product inoculation

Yeast-like growth was induced by inoculation of hypha/spore suspension into sealed yogurt packages which contained headspace compositions sufficient to induce a morphological switch as described in section 2.2. These conditions most closely mimicked real-world product conditions and allowed for the quantification of headspace compositional changes in the challenge study. For challenge studies, inoculum was added to lactic acid bacteria (LAB) containing skim milk that was then transferred to retortable plastic bags and heat-sealed (retortable pouches, Amcor Limited, Hawthorne, Victoria, Australia). Bags were incubated at 42°C for 8 h to allow for LAB fermentation of the milk, similar to continuous production in cup fermentation. Yogurt was subsequently transferred to 4°C, 15°C, or 25°C for the remainder of shelf-life.

For determination of visible package bloating, rubber septa were used to prevent the disturbance of the headspace in the yogurt cup. Studies quantitatively comparing the yeast spoilage among the isolates discussed in Table 1 were initially performed by inoculation of commercially purchased 150.25 g (5.3 oz) yogurt cups and incubation of yogurt in a GasPack anaerobic chamber jar. However, minimal proliferation was observed among all isolates (data not shown; similar trends shown in Figure 4). Carbon dioxide production and package bloating occurred under these same conditions, independent of yeast-like proliferation. Therefore, "days until visible container bloating" was reported in Table 1 and was performed, as described in section 2.2, by inoculation into a sealed yogurt cup instead of in an anaerobic chamber jar. Use of an anaerobic chamber jar would not have allowed for sensitive quantification of headspace changes. These conditions are additionally less representative of commercial packaging. For these reasons, individual packaging of yogurt samples which allowed for headspace

analysis and package destruction at every sampling point in the challenge study was performed.

Challenge study treatment conditions

Natamycin was added in the form of commercially available Natamax (DuPont USA, Madison, Wisconsin). Natamax is composed of 50% lactose, which serves as a carrier. Final concentrations of natamycin in yogurt or milk were 0, 5, 10, 15, and 20 ppm (μg/ml). Initially, 10x solutions of natamycin in milk were prepared, then volumetrically transferred to yogurt/milk. Yogurt/milk was then aliquoted into individual sample bags before transfer to storage. For the cup fermented yogurt used in the yeast challenge study, Day 0 sampling was done immediately following the 8 h fermentation at 42°C. Natamycin was added to the retortable bag before the 8 h fermentation. Microbial analysis immediately before and immediately following the 8 h fermentation showed no fungal proliferation or death.

Microbial analysis

Yogurt samples were analyzed at Day 0, 3, 7, 14, 21, and 28 for the growth curve comparison among various *M. circinelloides* isolates. For the challenge study, samples were analyzed at Day 0, 3, 7, 14, 22, and 30. At each sampling point, a new yogurt bag was analyzed and destroyed. Microbial counts were determined by plating on acidified PDA and incubating plates aerobically at 25°C for 48 h, regardless of challenge study conditions

Headspace analysis

Retortable plastic bags containing cup-fermented yogurt were analyzed at every

sampling point in the challenge study with yeast-like cells as described in section 2.4. Bags were fitted with adhesive-backed rubber septa and a Mocon headspace analyzer (MOCON Inc., Minneapolis, MN) was used to quantify the percent oxygen and carbon dioxide within the package. Prior to recording experimental data, atmospheric conditions were measured as a control.

Thermal destruction

Changes in microbial counts were recorded for fungal cultures suspended in milk and incubated in water baths of varying temperatures. Methods were performed as previously described (Splittstoesser et al. 1996, Usaga et al. 2014), with the exception of using skim milk as the medium during thermal treatment and pour plating survivors onto acidified PDA. Briefly, either yeast-like or hyphal phase cells were scraped from acidified PDA plates and re-suspended in skim milk. Cell suspension (20 µl) were injected into glass capillary tubes (1.5 to 1.8 by 100 mm; Kimble Chase, Vineland, NJ) using a syringe fitted with a repeat dispenser (Hamilton Co., Reno, NV) and glass capillary tubes were heat sealed. For each time point sampled, five replicate capillary tubes were prepared for a total of 100 µl volume of cell suspension. Non-treated controls were included as "time 0" data points. Capillary tubes containing hyphal phase cells were incubated in water baths at temperature 54°C, 56°C, and 58°C. Capillary tubes containing yeast-like cells were incubated in water baths at temperature 51°C, 53°C, and 55°C. Sampling points were recorded at intervals appropriate to the rapidity of cell death for a given temperature.

At selected time points, capillary tubes were removed from the water bath and immediately submerged in 70% ethanol on ice. Surface-sterilized capillary tubes were

blotted, transferred to milk dilution bottles containing 20 ml peptone water, and crushed using a glass rod. Subsequent serial dilutions were plated using acidified PDA. Plates were incubated at 25°C for 48 h, then enumerated.

Microbial count calculation

Microbial counts were determined at each sampling point in the challenge studies by enumeration of growth on acidified PDA plates. Counts were transformed into log numbers and plotted vs time. Standard deviation is shown for each time point, for which counts were performed in triplicate. Count data were similarly analyzed in Figure 1, but to account for variability in the initial counts in scraped plate inoculum, data were normalized by reporting count increases compared to the initial population at time 0.

Thermal destruction

Thermal destruction curves were constructed by plotting the log number of fungal survivors vs the length of time in the hot water bath. *D*-values were determined as the negative inverse of the slope of the thermal destruction curves. *D*-values for each morphology, hyphal and yeast-like phases, were determined for three temperatures. The log of these three *D*-values were plotted against temperatures and the negative inverse of the slope of the resulting line of best fit was the calculated *z*-value. Experiments were performed in triplicate and all linear coefficients of determination (r^2) were >0.90.

Statistical analysis

Statistical analyses were performed on challenge study data at a given temperature for both yeast-like and hyphal phase cells. Four-way ANOVAs were

performed using a procGLM model (SAS Institute Inc., Cary, NC). The alpha levels was set at 0.05 and differences were considered significant at a *P* value of < 0.005 which included a Bonferroni correction for multiple comparisons.

Microscopy

Imaging was conducted using a bright field microscope (Axiophot, Zeiss, West Germany). Hyphal microscopy included in Figure 2a was assembled from a series of three static images captured at 2 μm vertical intervals using focus stacking software at 400x magnification (Helicon Focus, Helicon Soft Limited, heliconsoft.com). Yeast-like and hyphal phase specimens were stained with cotton blue in lactoglycerol (Becton, Dickinson and Co., Franklin Lakes, NJ) prior to wet mounting on a microscope slide. Microscopy was also utilized during experimental work to confirm the morphology of the cells used in analysis and cells were prepared the same as above.

4.3 Results

Spoilage potential in the hyphal phase

The yogurt spoilage isolate of *M. circinelloides* increased ~3 log CFU/g compared to the 0 to 1 log CFU/g proliferation of other *M. circinelloides* isolates from environmental, industrial, and clinical sources. The aerobic growth of foodborne, clinical, and industrial strains of *M. circinelloides* in yogurt is shown in Figure 1. Under aerobic conditions, *M. circinelloides* grows in the hyphal phase, and fungal growth is characterized by visible mycelial development on the yogurt surface. At 25°C, hyphal phase growth was initiated within three days of incubation. Additionally, maximum population increases were achieved by day 14. The spoilage isolate showed an increased

ability to proliferate in yogurt relative to other isolates sourced from clinical infections, environmental sources, and industrial fermentations. This proliferation is indicative of the severe quality changes associated with 2013 product recall and underscores the importance of utilizing food isolates in challenge studies, as opposed to other laboratory strains.

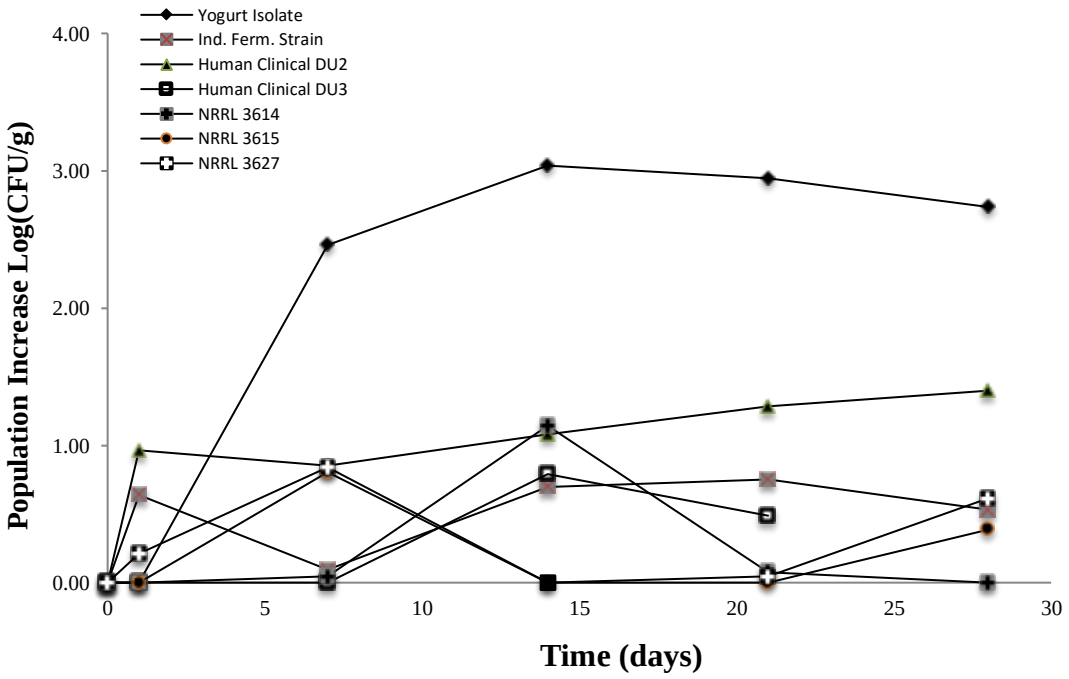


Figure 4.1: Aerobic, hyphal phase growth of clinical isolates, industrial strains, and the yogurt spoilage isolate in yogurt stored at 25°C.

Spoilage potential in the yeast-like phase

Many of the changes in quality reported during the 2013 product recall are specific to yeast-like phase growth of *M. circinelloides.* Carbon dioxide production resulted from yeast-like growth, which we were able to induce by inoculation of sealed cups of Greek yogurt through a rubber septum. All of the isolates were able to produce container bloating within three days at 25°C and production of CO_2 was confirmed by

headspace analysis. No mycelial development was observed in inoculated yogurt cups incubated at 25°C, spoilage resulted from visible container bloating. The yogurt spoilage isolate, along with an isolate from pig manure obtained from the NRRL, were able to do so within 24 hours. Under the yeast-like morphology inducing conditions, there is limited proliferation among all isolates (data not shown; similar trends to yeast-like growth displayed in Figure 4). However, despite negligible increases in counts, CO_2 production (i.e. visible container bloating) was still detected and lead to product spoilage.

Table 4.1: Reduced oxygen, yeast-like phase CO_2 production in yogurt cups by clinical isolates, industrial strains, and the yogurt spoilage isolate in yogurt stored at 25°C.

Mucor circinelloides **strain**	**Source**	**Days until yeast-like phase incubation induced visible yogurt cup bloating**
Yogurt Isolate	Recall-Associated Yogurt Spoilage Isolate (Duke University, US)	1
Industrial Fermentation	(Nanjing, China)	3
Human Clinical DU2	Human Skin Isolate (Duke University, US)	2
Human Clinical DU3	Human Mucosal Isolate (Duke University, US)	3
NRRL 3614	Pig Manure (Netherlands)	1
NRRL 3615	Frozen Beef (Germany)	2
NRRL 3627	Iguana Lung (Netherlands)	3

Hyphal phase thermal resistance

The thermal resistance of the *M. circinelloides* yogurt spoilage isolate was assessed in both the hyphal and yeast-like growth phases. Hyphal phase growth was

confirmed by colony and cell morphology (Figure 2A) and was characterized by aerial mycelial development on plates incubated under aerobic conditions. Microscopic examination of these cultures revealed the typical branched, aseptate, apically growing hyphae (Schipper 1976; Pitt and Hocking, 2009). Hypha and asexual spores were observed by microscopy before thermal destruction. In skim milk as a medium, destruction of the hyphal phase cells was readily achieved at 58°C. At 58°C, about a log of hyphal phase *M. circinelloides* was eliminated every two minutes. The *D*- and *z*-values for hyphal phase cells (Table 2) indicate that cells would be readily eliminated under typical milk pasteurization regimes (ex. 72°C for 15 seconds) (Codex 2004).

Yeast-like phase thermal resistance

The *M. circinelloides* yogurt spoilage isolate in the yeast-like phase was determined to be less resistant to thermal treatment than hyphal phase cells. Yeast-like growth was confirmed by colony and cell morphology (Figure 2B). Plates incubated under anaerobic conditions yielded smooth, round colonies characteristic of yeast. Microscopic examination revealed small, round cells indicative of isotropic growth. In the yeast-like phase, cells were rapidly destroyed by treatment at 55°C using skim milk as a medium. Relative to hyphal phase cells, inactivation of hyphal phase cells required less exposure to thermal processing (Table 2).

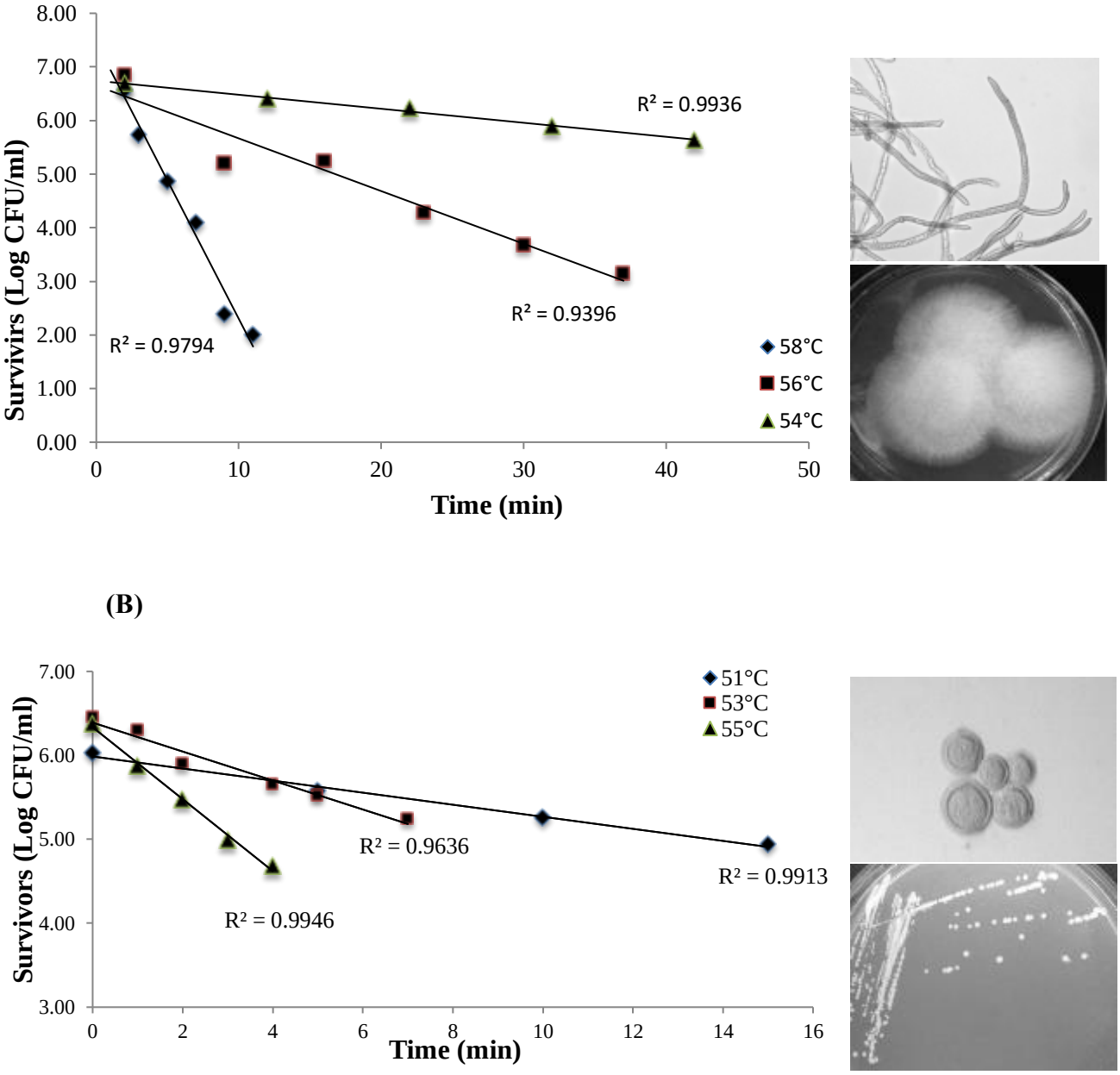


Figure 4.2: Thermal destruction curves for hyphal phase (A) and yeast-like phase (B) *Mucor circinelloides* yogurt spoilage isolate in skim milk. (n=3). Embedded images show cellular and colony morphologies in the hyphal and yeast phases. Microscopic images are 400x magnification.

Table 4.2: The *D*- and *z*-values for hyphal phase and yeast-like phase *Mucor circinelloides* yogurt spoilage isolate in skim milk.

Hyphal Phase	***D*-value (min)**	***z*-value (°C)**
58°C	1.94 ± 0.53	3.09
56°C	10.17 ± 0.28	
54°C	38.31 ± 0.02	
Yeast-like Phase	***D*-value (min)**	***z*-value (°C)**
55°C	2.44 ± 0.35	0.34
53°C	6.87 ± 1.19	
51°C	14.25± 0.12	

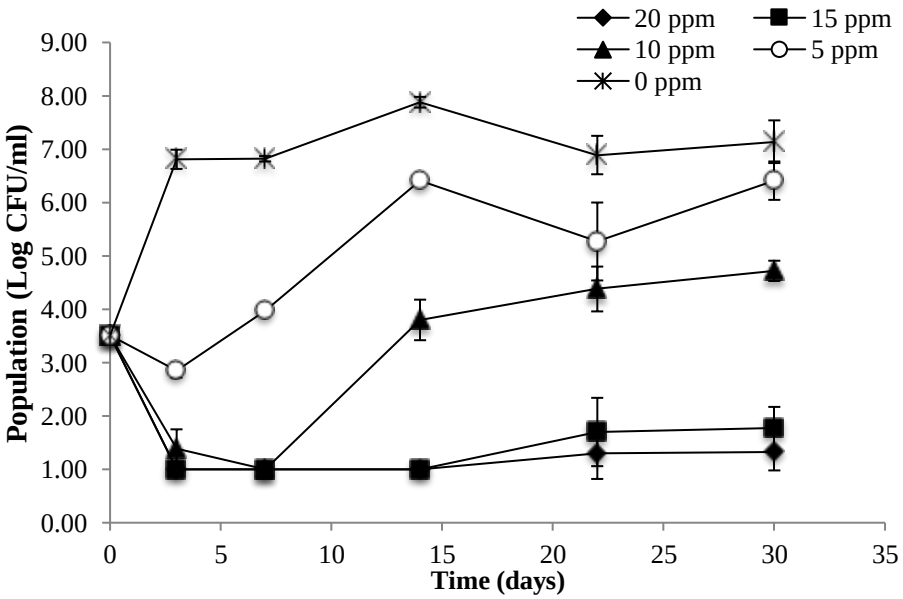
Challenge study

The impact of the antifungal additive, natamycin, at varying concentrations on yogurt spoilage was evaluated by assessing fungal growth in the hyphal phase, yeast-like phase, and by measurement of CO_2 production in the yeast-like phase. Yogurt was incubated at 4°C, 15°C, 25°C and the effect of temperature abuse on spoilage is an indication of the storage conditions which lead to the large-scale recall.

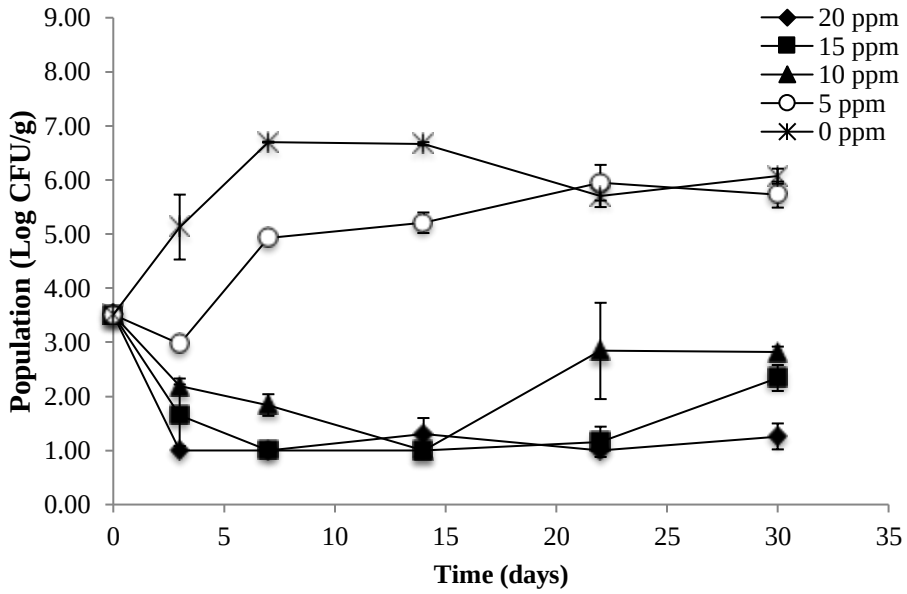
Hyphal phase growth under various incubation conditions is shown in Figure 3. The maximum population increase achieved under each storage condition was ~3.5 log CFU/g yogurt. Spoiled yogurt had visible mycelial growth and syneresis. However, the speed at which maximum counts were achieved decreased as storage temperature increased. Additionally, the relative effectiveness of natamycin addition was impacted by storage temperature. At storage temperatures of 4°C and 15°C, 10 ppm (was sufficient to significantly inhibit hyphal phase growth throughout the 30 day incubation period. When storage temperature was increased to 25°C, outgrowth of fungi was observed by day 14 with 10 ppm natamycin. However, at 25°C, natamycin

concentrations of 15 ppm or greater were necessary to inhibit hyphal growth. Natamycin at a concentration of 10 ppm significantly reduced fungal proliferation compared to those treated with 0 ppm or 5 ppm natamycin. However, response to natamycin concentrations of 15 ppm and 20 ppm were significantly different compared to 10 ppm natamycin concentrations by day 30 of the challenge study. Fungacidal activity was observed at natamycin concentrations in excess of 5 ppm. Die off of *M. circinelloides* was more rapid at 15°C and 25°C, although the counts were eventually reduced to below detection limits under all incubation conditions. Though, natamycin concentrations of 10 ppm allowed hyphal phase populations incubated at 15°C and 25°C to recover before the end of the 30 day incubation period.

(A)



(B)



(C)

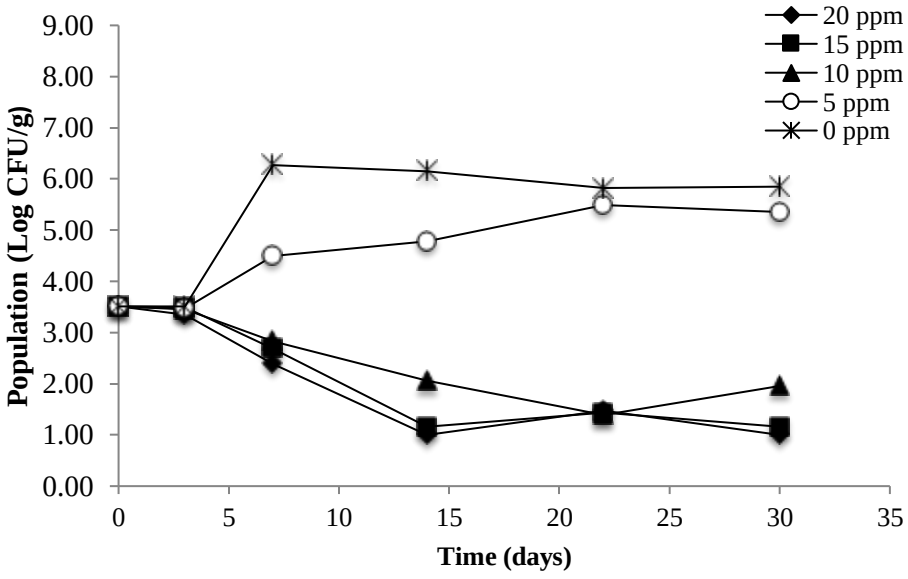


Figure 4.3: Hyphal phase growth with various natamycin concentrations over 30 days of storage at: (A) 25°C, (B) 15°C, and (C) 4°C.

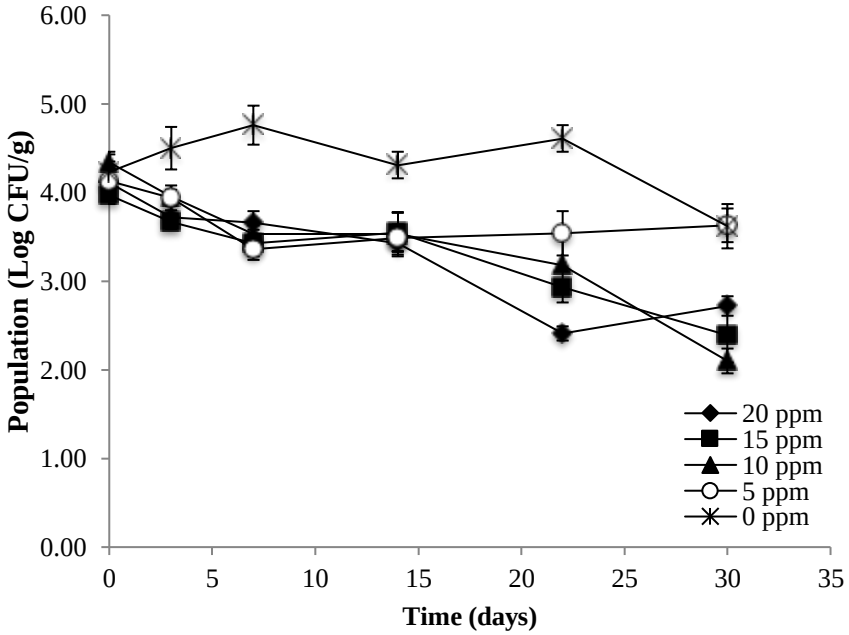
Yeast-like phase

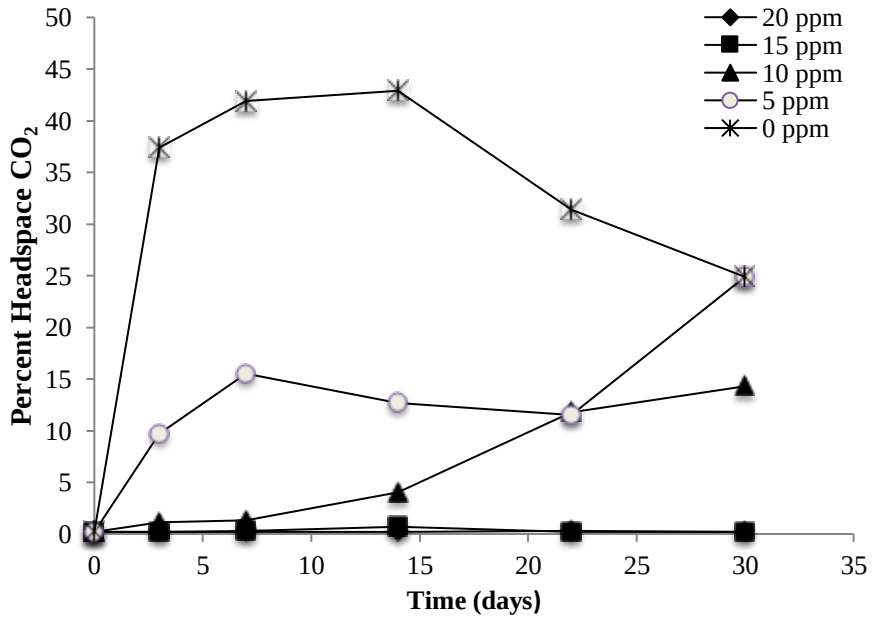
Successful preservation in the yeast phase challenge study was assessed by fungal proliferation as well as by measurement of CO_2 production via head-space analysis. As previously mentioned, minimal growth was observed in the yeast-like phase, despite rapid CO_2 production (Figure 4). Subsequently, natamycin was not essential in preventing outgrowth of *M. circinelloides* in the yeast-like phase, but because CO_2 production was detected in yogurt containers stored at 15°C and 25°C, natamycin was necessary to prevent container bloating. Production of excessive CO_2 was not, however, detected in yogurt containers incubated under strict temperature control at 4°C. This suggests that some temperature abuse is required to induce CO_2 production associated with commercial spoilage.

As with the hyphal phase challenge study, the concentration of natamycin to prevent CO_2 production was also dependent on the degree of temperature abuse. At 15°C, 10 ppm of natamycin was sufficient to control CO_2 production throughout a 30 day incubation period. Natamycin at 5 ppm controlled CO_2 production for 14 days at 15°C, but CO_2 levels had a moderate increase (~10%) by day 30. At 25°C, 15 ppm natamycin was necessary to control CO_2 production by yeast-like phase *M. circinelloides*. Without any antifungal in place, CO_2 production occurred rapidly. CO_2 levels reached almost 40% of the total headspace by day 3 of incubation at 25°C, despite the limited growth observed in the yeast-like phase. Natamycin yielded a modest fungicidal effect at 25°C, reducing the counts by about 2 log with 10 ppm natamycin or greater. However, this same fungicidal effect was not apparent in yogurt

incubated at 4°C and 15°C, suggesting that refrigeration provides some protective affect and increases *M. circinelloides* survival.

(A)





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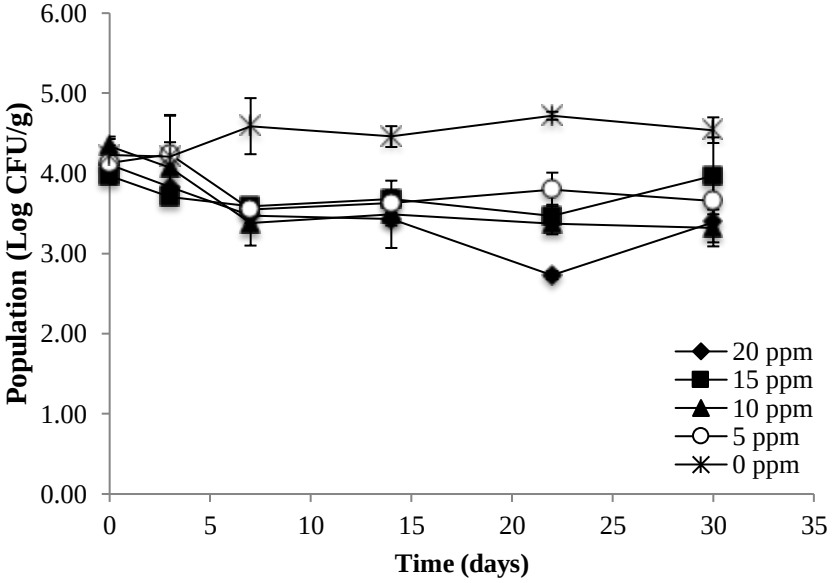
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Population (Log CFU/g)
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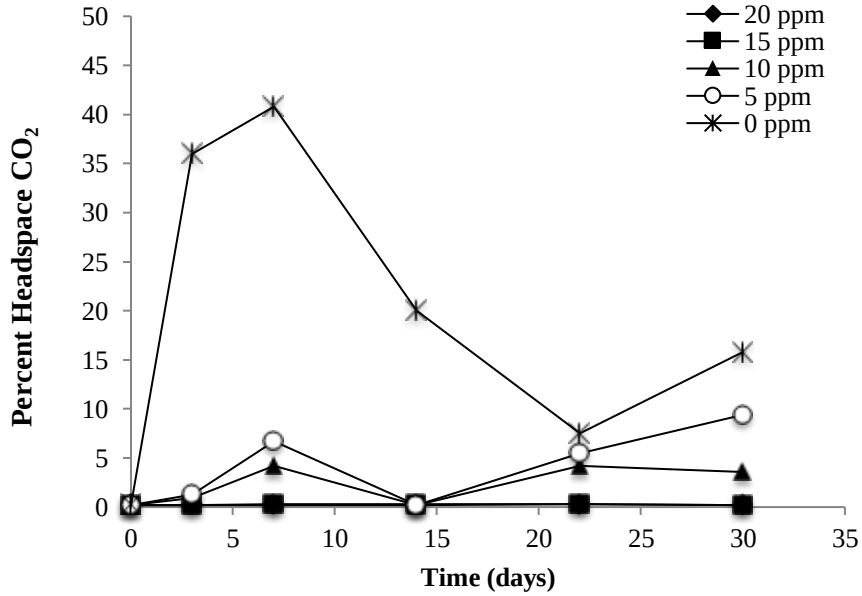
15 ppm
10 ppm
5 ppm
0 ppm


50
45
40
35
30
25
20
15
10
5
0
Percent Headspace CO2
0
5
10
15
20
25
30
35
Time (days)
20 ppm
15 ppm
10 ppm
5 ppm
0 ppm

(C)

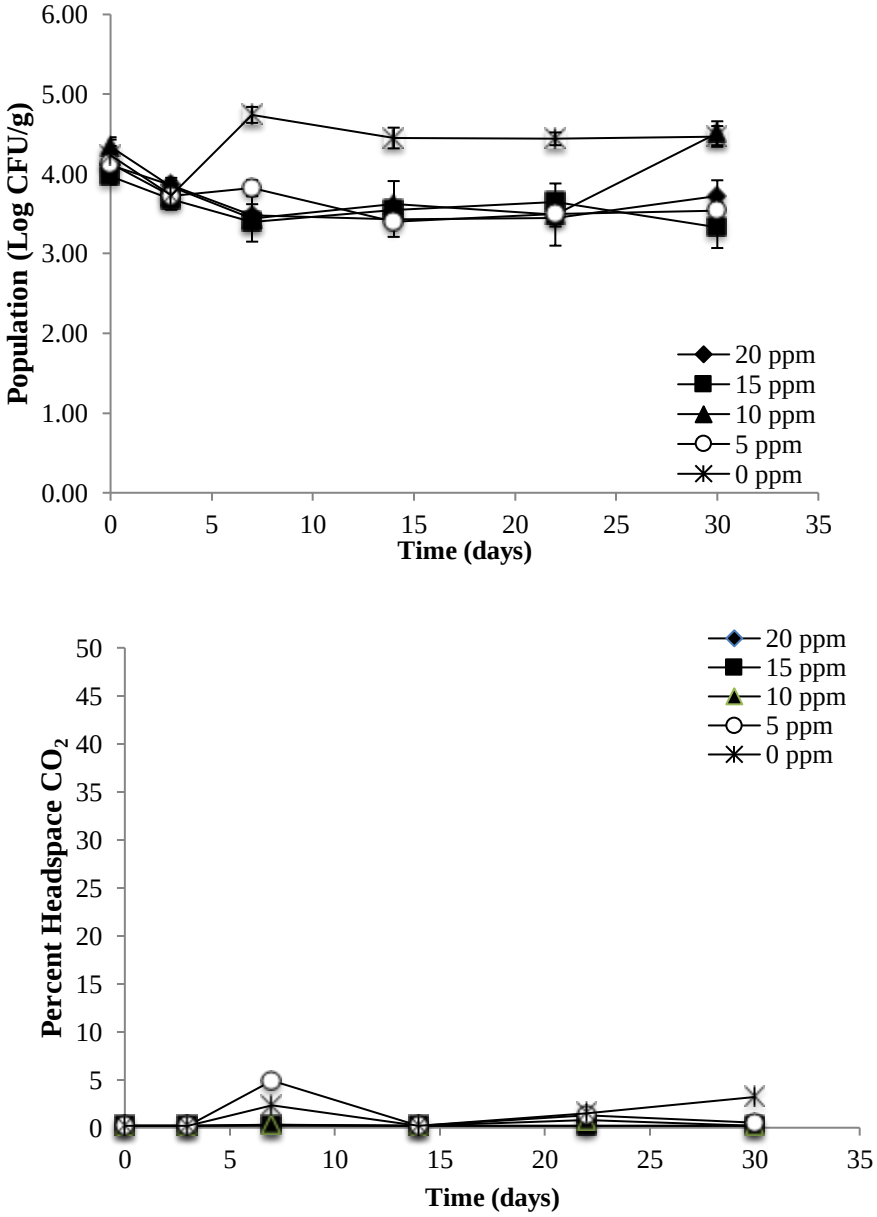


Figure 4.4: Yeast-like phase growth and carbon dioxide production under various natamycin concentrations over 30 days of storage at: (A) 25°C, (B) 15°C, and (C) 4°C.

4.4 Conclusions

The recall of commercially produced yogurt by *M. circinelloides* represented a

new and unusual spoilage incident. Most *Mucorales* are saprobic and are found in decaying organic matter (Alexopoulos et al. 1996). *M. circinelloides* is a common secondary plant pathogen, causing spoilage of fresh fruits and vegetables (Jones and Aldwinckle, 1990). Its contribution, though, to spoilage of processed products is unusual, due in large part to the obvious quality deterioration (bloating and bubbling of containers) associated with yeast-like CO_2 production. The process of contamination and spoilage has not been studied in this fungus, which is distantly related to many of the better-studied Ascomycetes (*Penicillium, Aspergillus, Saccharomyces)* widely associated with food spoilage (Alexopoulos et al. 1996; Ray and Bhunia 2008; Samson et al. 2010).

The yogurt spoilage isolate of *M. circinelloides* showed an increased spoilage potential compared to environmental and clinical isolates of *M. circinelloides*, as shown in Figure 1 and Table 1. The genetic determinants for this increased fitness are unknown, but genomic analysis has indicated that this isolate has a surprising number of genes associated with the synthesis of secondary metabolites when compared to other species of early divergent fungi (Lee et al. 2014; Keller et al. 2005). This comparison underscores the importance of using food-sourced isolates in challenge studies and characterization work in order to draw conclusions that are relevant in food applications (Usaga et al. 2014).

The thermal resistance of *M. circinelloides* is an indication of whether or not this organism resulted from post-processing contamination or, if like some Ascomycetes (*Byssochlamys, Aspergillus, Talaromyces*) that form heat resistant ascospores, it can survive the pasteurization process and cause spoilage later in shelf-life (Murdock and

Katcher 1978; Splittstoesser, D and Splittstoesser C 1977; Beuchat 1986). The hyphal phase and yeast-like cells were not shown to be particularly heat resistant. Two other forms of *M. circinelloides* exist, sexual spores and asexual resting spores, neither of which were specifically evaluated in the thermal destruction work in this study. Chlamydospores and arthrospores are thickly encysted cells that form rarely, usually near the growth substrate (Trinci and Collinge 1974; Hesseltine et al, 1985; Sonneborn et al. 1999). These tend to be more resistant forms generated within a small subset of the hypha (Barrera 1983; Hibbett et al. 2007; Schipper 1976). Our microscopic evaluation indicated that arthrospores were present, but were likely only a very small percentage of the total hyphal phase population harvested for thermal destruction (Figure 5). Additionally, zygospores only form when two compatible mating pairs of this heterothallic fungus meet under the appropriate environmental conditions (Choppin et al. 1997; Wurtz and Jockusch 1975). Based on these observations, it is unlikely that *M. circinelloides* survived the pasteurization process and is instead a contaminant likely to have been introduced after thermal treatment.

Figure 4.5: Arthrospore formation in hyphal phase cells of the *Mucor circinelloides* yogurt spoilage isolate. Image is 400x magnification.

The hyphal phase cells were found to be more heat resistant than yeast-like cells, which is consistent with literature reports that have identified heat resistant hyphal phase cells, but no thermoresistant yeast phase fungi, is relevant to food spoilage (Beuchat 1986; Murdock and Hatcher, 1978; Splittstoesser D. and Splittstoesser C. 1977; Tournas 1994). It is also likely that the original contamination in the processing facility occurred from hyphal phase cells. Once conditions in the packaged container became amenable to yeast-like growth because of the residual metabolic activity of the lactic acid bacteria, CO_2 production resulted from *M. circinelloides*. Incubation of the milk with *M. circinelloides* alone, or incubation of milk with lactic acid bacteria alone did not result in CO_2 production. The presence of both lactic acid bacteria and *M. circinelloides* was required in order to achieve the characteristic package bloating observed by consumers. Previous reports of *Mucor* spoilage in fermented foods have similarly stated that CO_2 production by the intentionally inoculated fermentative organisms resulted in yeast-like growth and container bloating (Foschino et al., 1993; Hesseltine, C., et al. 1985).

Natamycin is a polyene macrolide antifungal synthesized by *Streptomyces natalensis* and has been on the U.S. market as an approved additive for decades (Aparicio et al. 2015; Thomas and Broughton, 2001). The European Food Safety Authority issued an opinion in 2009 on the safety of natamycin used as a preservative on cheese surfaces stating that the low concentration needed to achieve preservation provided an adequate margin of safety (EFSA 2009). In the U.S., it was originally approved for use at concentrations up to 20 ppm in cheese and has been used

extensively to prevent mold growth on the surface of shredded cheese (CFSAN 2015). Recently, the Pasteurized Milk Ordinance allowed for the addition of antimicrobial additives to yogurt without violation of the product's standard of identity (CFSAN 2005). In 2014, the company DSM Food Specialties (Heerlen, Netherlands) submitted a letter of GRAS notification to the FDA for the use of 5 ppm natamycin in yogurt (CFSAN 2014). The FDA has since issued a letter of no objection to this usage (CFSAN 2015). Based on the results of this study, 5 ppm of natamycin is sufficient to inhibit yeast-like CO_2 production with moderate temperature abuse. Similarly, a challenge study published by El-Diasty et al. (2009) reported that 10 ppm natamycin inhibited *Mucor* proliferation in yogurt stored at 4°C, and 10 ppm was the lowest concentration evaluated. The results of the current study, however, indicate that temperature abuse impacts the efficacy of natamycin against *M. circinelloides* spoilage. Hyphal phase growth or severe temperature abuse require an increased level of natamycin in order to control spoilage by *M. circinelloides*, although the extreme temperature abuse shown in part (A) of Figures 3 and 4 is unlikely to occur.

Despite the GRAS status, many yogurt produced today do not utilize preservatives like natamycin and microbial spoilage issues occur at a relatively low rate. The utility of preservatives is dependent on the production conditions specific to individual manufacturers, as well as their tolerance for and baseline level of environmental contamination. Other mitigation strategies exist to control for *M. circinelloides*. As evidenced by the findings reported here, limiting post-processing contamination is the most obvious way of preventing spoilage, which may include protected packaging areas, various sanitation regimes, and environmental monitoring

(Samson et al. 2010). Srict maintenance of the cold chain is also an effective control for the CO_2 production reported by consumers. This strategy, though, depends on consumer behavior which can be difficult to ensure will be adequate to limit spoilage (Almonacid-Merino and Torres, 1993; Wordsfold and Griffith, 1997). The challenge study data presents the intersecting impact of storage temperature and natamycin concentration on *M. circinelloides* spoilage of yogurt. Taken together, this information can be used to determine an appropriate prevention or intervention strategy depending on a given set of conditions or the degree of control a manufacturer wishes to have over *M. circinelloides* spoilage.

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CHAPTER 5

Interspecific and intraspecific spoilage potential variability among food-sourced *Alicyclobacillus* spp.

ABSTRACT

Alicyclobacillus spoilage represents an important quality threat for fruit juice manufacturers. Allelic associations between strain and isolation source have indicated that spoilage potential may vary by species, isolation source, or growth medium. However, the relationship between spoilage and these predictors has remained a confounding factor in characterization studies and quality control programs. Food sourced isolates (n53) were grown at 45°C and 30°C in PDB acidified to pH 3.5 with various organic acids associated with fruit juices. Growth (OD_{600}) and guaiacol concentration were recorded and whole genome sequencing was performed. Phylogenetic relationships among the isolates were parsed using a maximum likelihood tree based on core SNPs. Guaiacol concentration ranged from <0.1 to 7.86 ppb and was positively associated with rapid proliferation ($p = 0.017$), while an extended lag phase, even if an equivalent final density was achieved, was not significantly associated with guaiacol production. The growth medium did not impact proliferation, but citric acid negatively impacted guaiacol production compared to growth in malic or tartaric acid ($p = 0.027$). Individual strains varied greatly in guaiacol concentration under different growth conditions. Interclass correlation coefficients indicated a significant portion of the variability in guaiacol concentration was explained by strain behavior (36%). These

results indicate that while medium can be predictive of *Alicyclobacillus* spoilage, species and isolation source are not strongly correlated with guaiacol production. This would suggest that spoilage potential may be a physiological state, dependent on the environment, more than a static trait of the isolate.

5.1 Introduction

Inter- and intraspecific variation in functional ability has been reported in bacteria. Prior to molecular subtyping techniques, variations in metabolism, salt tolerance, and oxidase reactivity were used in species and sub-species level identification. Among foodborne pathogens, variations in virulence factors impact pathogenesis (Johnson et al., 1988). Spoilage biology likewise varies within and between species (Snyder et al., 2016; Stohr et al, 2011). Additionally, a single species or strain may behave differently depending on the conditions to which it has been adapted (Usaga et al., 2016). In the first case, genetic differences which may or may not correlate with phylogeny dictate functional variation. In the latter case, the environment dictates phenotype. Variation in functional ability impacts the utility of detection methods which exclusively focus on presumptive identification. Quality assurance programs are often based on risk assessments for specific threats. Subsequently, over sensitive detection methods which target the presence of organisms that are not strongly associated with functional outcomes have decreased utility. These generalized detection methods may function as indicators of ingredient quality, but may not be predictive of spoilage potential or aid in product disposition decisions. Overzealous disposition strategies may increase waste in the food industry or decrease the value of the product

based on customer specifications which may not be reflective of product quality.

Alicyclobacillus spp. are food spoilage bacteria ubiquitously found in the soil and pre-harvest production environments. Recently, natural flavorings have been identified as a source of *Alicyclobacillus* in many diverse products (Oteiza et al., 2014). *Alicyclobacillus* is particularly challenging in fruit juice production because it is an acidophilic spore former. In conventional juice production, the thermal pasteurization process inactivates vegetative bacterial cells and the product's natural acidity inhibits the outgrowth of survivors (Bevilacqua et al., 2008). *Alicyclobacillus* spores, in contrast, survive the pasteurization process and germinate in the acidic medium (Silva et al., 1999).

Spoilage is typified by off-aromas associated with guaiacol (2-methoxyphenol) and the halophenols 2,6-dibromophenol and 2,6-dichlorophenol which are produced by *Alicyclobacillus* vegetative cells. The retronasal sensory threshold for guaiacol is 2.23 ppb in a room temperature juice matrix for trained panelists, while the taste threshold approaches 50 ppb (Orr et al., 2002; Kilcast, 2010). Spoilage is usually associated with off aromas, rather than turbidity or other visual defects, making quality failures hard to detect. *Alicyclobacillus* proliferates optimally under elevated temperatures and acidic conditions (Goto et al., 2003). Standardized methods recommend incubation of *Alicyclobacillus* spp. on media acidified to a pH of 3.5 at ~45°C (Isabel and Rolenda, 2000). Under normal storage conditions of shelf-stable juices and beverages, *Alicyclobacillus* proliferates slowly and detectable spoilage emerges over weeks or even months, often without appreciable growth. Because initial contamination occurs at low levels, current detection methods involve filtration of large volumes of product (up to 1

L) and plating on selective media. However, these detection methods do not consider the relative ability of *Alicyclobacillus* strains to germinate and variably cause spoilage in different juice and beverage products.

Two primary species of Alicyclobacilli have been isolated from juice products, *Alicyclobacillus acidoterrestris* and *Alicyclobacillus acidocaldarius*. Durak et al., (2010) demonstrated that *Alicyclobacillus acidoterrestris* is more frequently isolated from apple juice, while *Alicyclobacillus acidocaldarius* is more frequently isolated from orange juice. Witthuhn et al. (2013) identified species-level differences in spoilage potential and reported increased guaiacol synthesis at lower temperatures in *A. acidoterrestris* strains compared to *A. acidocaldarius* strains grown in apple juice. Most studies have only evaluated *A, acidoterrestris* even though both species have been isolated from production and processing environments (Groenewald et al, 2009). Diversity in spoilage potential has also been observed at the subspecific level (Ciuffreda et al., 2015). Additionally, Goto et al. (2007) reported variations in the growth of a single strain among different juice products, depending on the type of juice. The objective of this study was to assess variability in spoilage potential, as determined by level of guaiacol production, among a diverse collection of food-sourced isolates to determine predictors associated with spoilage.

5.2 Materials and methods

Bacterial isolates

Alicyclobacillus isolates from juices and juice production environments were selected from an existing collection (Durak et al., 2010). In total, 53 non-redundant *Alicyclobacillus* isolates were collected by filtering contaminated product (500 ml)

through a 0.22 μm sterile cellulose filter (Millipore, Bedford, MA). The filter was transferred to potato dextrose agar (PDA; BD Difco, Sparks, Maryland) acidified to pH 3.5 with tartaric acid. Colonies from the filters were re-streaked for isolation, inoculated in potato dextrose broth (PDB; BD Difco, Sparks, Maryland), and incubated for 48 hours at 45°C with agitation at 225 rpm. Freezer stock of *Alicyclobacillus* spp. was made by adding 500 μL of the overnight culture to 500 μL of 50% glycerol in a 2 ml cryovial and stored at -80°C. Glycerol stocks frozen at -80°C were thawed immediately before use. The cultures were streaked onto PDA and incubated for 48 hours at 45°C.

Enumeration by Optical Density (OD) measurements

Growth was assessed in a model system by OD at 0, 8, 24, 48, and 72 hr when incubated at 45°C and every 24 hr for seven days when incubated at 30°C. Model systems were prepared by the acidifying PDB to pH 3.5 with one of three juice-relevant organic acids - tartaric, malic, and citric. Stock *Alicyclobacillus* cultures were streaked onto acidified PDA as described above and incubated at room temperature for two weeks. The plates were flooded with phosphate buffer, the suspended culture transferred to microfuge tubes, and heat treated for 20 min at 80°C. PDB was inoculated with heat-shocked spore culture (100 μL) and incubated with shaking at 225 rpm. OD was recorded at 600 nm (Milton Roy, Spectronic 20D+). Results of the OD levels were averaged across three biological replicates.

Growth curves were classified into one of three categories based on proliferative ability. These are presented graphically in supplementary materials (Tables S1 and S2)**.** The growth categories were: Low Growth Rate, Medium Growth Rate, High Growth Rate which were assigned based on the inflection point and the maximum OD achieved

at the end of the incubation period. Inflection was defined as the time point when the microorganism started to grow exponentially. The difference in OD between adjacent time points were calculated and the time point at the start of the greatest difference was the selected as the point of inflection. Therefore, isolates that proliferated rapidly to a high final density were categorized in High Growth Rate, isolates that proliferated more slowly but eventually reached a high final density were categorized as Medium Growth Rate, isolates which never achieved a high final density were categorized as Low Growth rate.

Guaiacol Detection

Guaiacol production was assayed using the peroxidase method as adapted from Bahceci and Acar (2007) and performed in a 96-well plate at the conclusion of a seven-day growth study at 30°C in PDB acidified to pH 3.5 with various organic acids. Samples were centrifuged (12,000 rpm for 10 min) and 30 µL was combined with 210 µL phosphate buffer (1/15 M, pH 6), 30 µL hydrogen peroxide (0.5%), and 5U peroxidase (Sigma-Aldrich, St. Louis, MO). Absorbance was immediately measured at 410 nm on a Synergy H1 spectrophotometer (BioTek, Winooski, VT) for both test samples and uninoculated controls. Concentration was determined using a guaiacol standard curve (Sigma, St. Louis, MO).

DNA extraction, library preparation, and Illumina sequencing

Alicyclobacillus gDNA was extracted using the QIAamp DNA MiniKit (Qiagen, Valencia, CA) as previously described (Jasna ref), but with an initial 1 hr incubation with 20 mg/ml lysozyme at 56°C to promote lysis. The concentration in the extracts was

normalized across samples to 0.2 ng/ μL using Qbit Fluorometric Quantitation (ThermoFisher, Waltham, MA). DNA libraries were prepared using a Nextera XT DNA Sample Preparation kit and Nextera XT Index kit (Illumina, Inc. San Diego, CA). Two of the isolates were sequenced on a MiSEQ using 2x250 bp paired-end rapid sequencing (Genomics Initiative, College of Veterinary Medicine, Cornell University) and the remaining 51 isolates were pooled and sequenced on a HiSEQ 2500 rapid run using 2x100 bp paired-end sequencing (Biological Resource Center, Cornell University).

Genome assembly and phylogenetic analysis

Illumina adapter sequences and low quality reads were removed using Trimmomatic v0.32 and subsequent read quality was evaluated using FastQC v0.11.2. with default parameters. High quality reads were evaluated using SPAdes v.3.8.0 with a k-mer length of 99 used in the analysis. In addition to the 53 isolates described above, open genomes for *Alicyclobacillus* type strains represented in the collection were extracted from the NCBI Genome database and included for comparison. These three strains were *Alicyclobacillus acidiphilus* 100859 (Matsubara et al., 2002), *Alicyclobacillus acidocaldarius* DSM 446 (Mavromatis et al., 2010), and *Alicyclobacillus acidoterrestris* ATCC 49025 (Shemesh et al., 2013). Other type strains for which the whole genome sequence was available were excluded due to the extreme diversity in genome size and content. These 56 genomes were used to construct a phylogeny based on core single nucleotide polymorphisms (SNPs) using kSNP v3 and a kmer size of 13. The core SNPs were used to build a maximum likelihood phylogeny in

RAxML under a general time-reversible model with gamma distributed sites (GTRGAMMA) and 1,000 bootstrap repetitions. The resulting tree was edited in FigTree v1.4.3 and Inkscape.

Statistical analysis

Statistical analyses were performed in RStudio (RStudio, Inc., Boston, MA). Growth curve data was transformed into proliferative categories as described above. An ANOVA was performed using the R package lmer for mixed effects models. Guaiacol concentration was modeled as a function of the fixed effects of proliferative ability, acidulant type, source of isolate, and species, as well as the random effect of isolate. A p-value < 0.05 was considered significant. The interclass correlation coefficient was calculated using the R package irr. The variability in guaiacol production for each strain under the three growth conditions was evaluated by determining the agreement using a one-way model.

5.3 Results

Our group has previously established species-specific associations with product isolation source (Druak et al., 2010), which suggests that particular species may be better adapted to spoilage of particular juice products. This is potentially supported by observations by Goto et al. (2007) that individual isolates were better able to propagate within apple juice compared to lemon juice blends. However, the medium itself may have a general impact on spoilage across isolates (Hsiaro and Siebert, 1999), or sub-species level variations associated with particular genotypes may be explanatory of

spoilage behavior. The collection of food-sourced *Alicyclobacillus* isolates was first grown in PDB at 45°C, conditions which are optimal for growth but which may not represent commercial spoilage scenarios. Isolates were categorized by their proliferative ability within a single growth medium (acidulant: malic, citric, and tartaric, or unacidified) and the proliferative ability for single isolates across different conditions is summarized in Fig. 1. Each isolate is represented once, based on its ability to proliferate readily in different media.

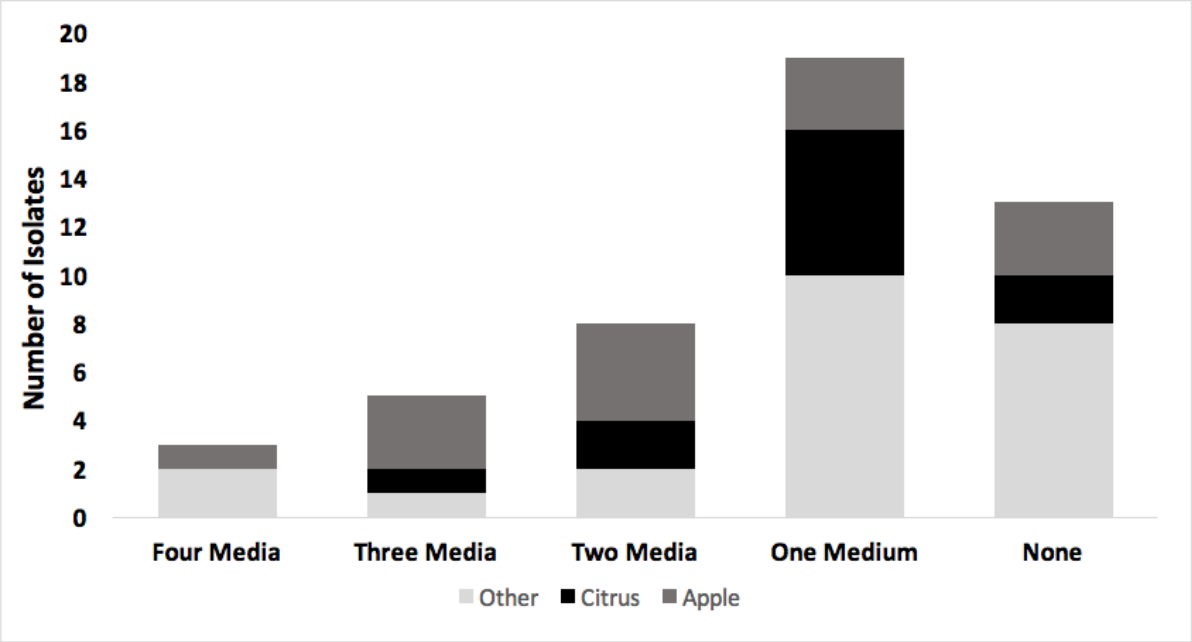

Figure 5.1: *Isolates with high proliferative ability in different media*. Each isolate is categorized by the number of growth conditions (PDB acidified with citric acid, malic acid, tartaric acid, or without acidification) in which it was grouped into the "High Proliferation" category. Bars are color blocked by isolation source of the isolates.

Isolates varied in their ability to replicate under different conditions, but the greatest number of isolates (n19) proliferated well in the presence of only one of the four acidulants. Fourteen isolates in the data set did not replicate efficiently in any of

the tested media, which indicated that these may not be likely to cause sensorial defects, or that their spoilage potential was not captured by this assay. The remainder of the collection was decreasingly capable of growing rapidly under multiple conditions. Orr et al. (2000) speculated that variability exists among strains for growth ability in product, but the relevance of proliferation on *Alicyclobacillus* is not clearly defined. Pettipher et al. (1997) reported a high level of growth in juice, although spoilage is defined by the production of off-aromas, as opposed to turbidity associated with high levels of growth. While guaiacol synthesis requires spore germination, guaiacol production often occurs without a quantifiable increase in the number of cells. However, studies have variably found associations between growth and guaiacol (Perez-Cacho et al., 2011).

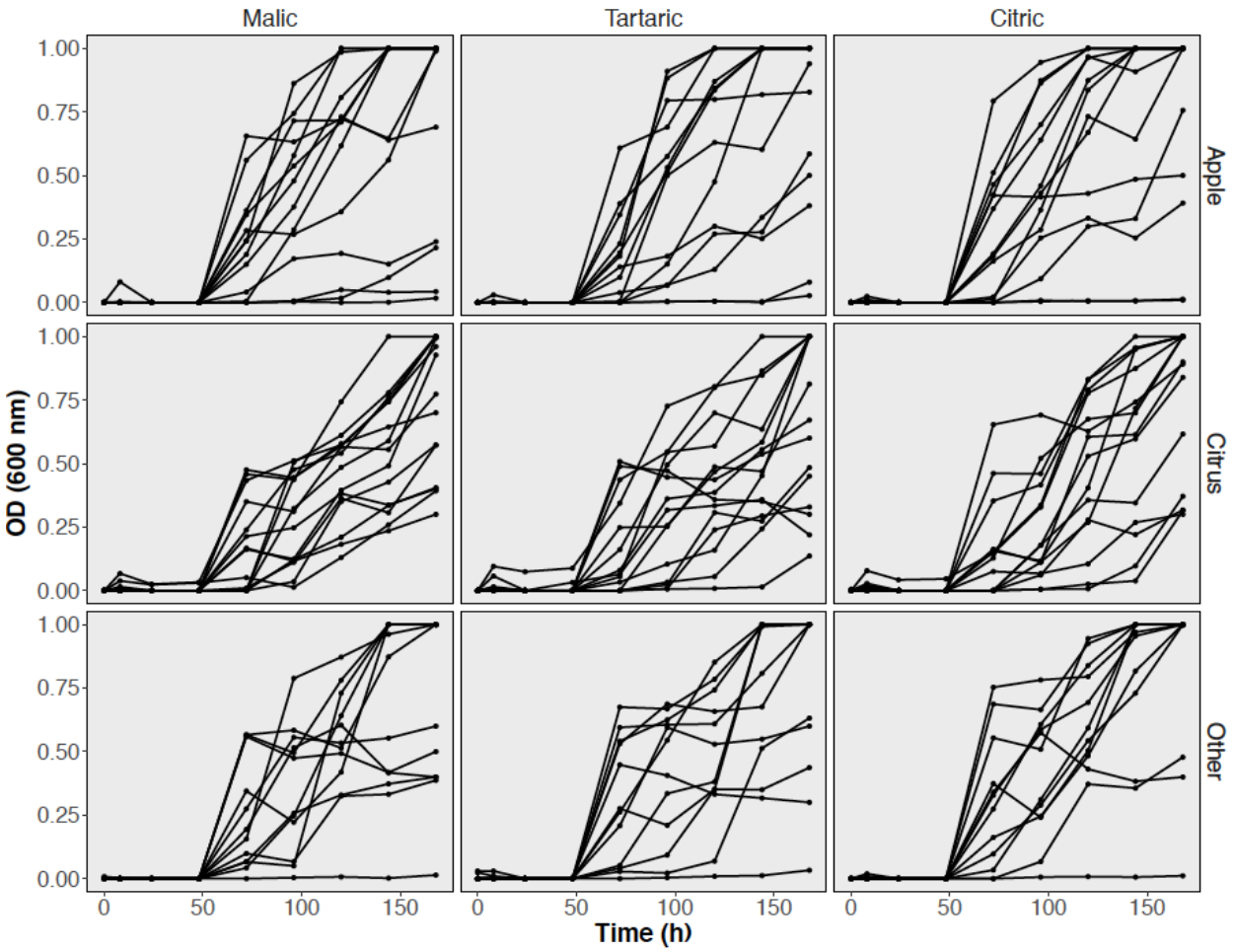

Figure 5.2: Growth curves for *Alicyclobacillus* spp. grown at 30°C in PDB for 168h. Columns represent the organic acid type used to acidify the medium (pH 3.5). Rows correspond to product isolation source. Growth is reported in optical density recorded at 600 nm, readings are the average of three replicates.

Given the thermophillicity of *Alicyclobacillus*, growth at ambient temperature incubation is an additional factor that impacts spoilage and reports of mesophilic growth has varied between and within species. Bahceci et al. (2005) noted that there was little growth in *A. acidoterrestris* at 25°C and no known growth by members of this species below 20°C. In fact, Witthuhn et al. (2011) observed up to a 2 log die off in juice stored below 25°C. Temperature ranges, along with pH ranges, which support growth vary by

species, and the interaction of temperature with species impacts guaiacol production (Witthuhn et al., 2013; Witthuhn et al., 2012). The culture collection was subsequently grown at 30°C to assess proliferative ability at temperatures which are more likely to occur during storage and distribution (Fig. 2). Isolates were capable of proliferating at 30°C, although the time for cultures to leave lag phase and reach stationary phase was necessarily extended. Individual strains performed better or more poorly depending on media, but growth potential was not effected by acidulant type overall. At the conclusion of the 168 h growth study at 30°C, cultures were evaluated for guaiacol production. Guaiacol has been variably associated with cell density or growth, strain, growth temperature, and oxygen temperature (Ciuffreda et al., 2015). The majority of previous studies have spiked the growth medium with 10-1,000 ppb vanillin, the precursor for guaiacol, to increase the final concentration (Molva and Baysal, 2016). Not surprisingly, the amount of vanillin introduced into the broth or juice directly impacts the final concentration of guaiacol synthesized. In juice systems which do not include artificially elevated levels of vanillin, the final concentrations of guaiacol synthesized by *Alicyclobacillus acidoterrestris* were 8.1 to 11.4 ppb in apple juice (Orr et al., 2000), 0.7 ppb in orange juice (Perez-Cacho et al., 2011), and 11-35 ppb in kiwi products (Zhang et al., 2013). The lower range for these values approach the limit of detection and recognition for guaiacol in fruit juice matrixes as identified by trained sensory panels. Therefore, the variation in the level of guaiacol synthesis may impact whether a contaminated product is considered unacceptable. In these experiments, only *Alicyclobacillus acidoterrestris* was evaluated as it is considered the primary causative agent of juice spoilage. Additionally, interspecific variation has not yet been addressed

on a large scale. In this study, guaiacol production was evaluated for 53 food-sourced isolates grown in media without supplementation of vanillin (Fig. 3). Although conclusions about the precise level and time frame for guaiacol development in juice products is limited due to the use of nutrient broth, intra- and interspecific variability can be directly compared across a large collection.

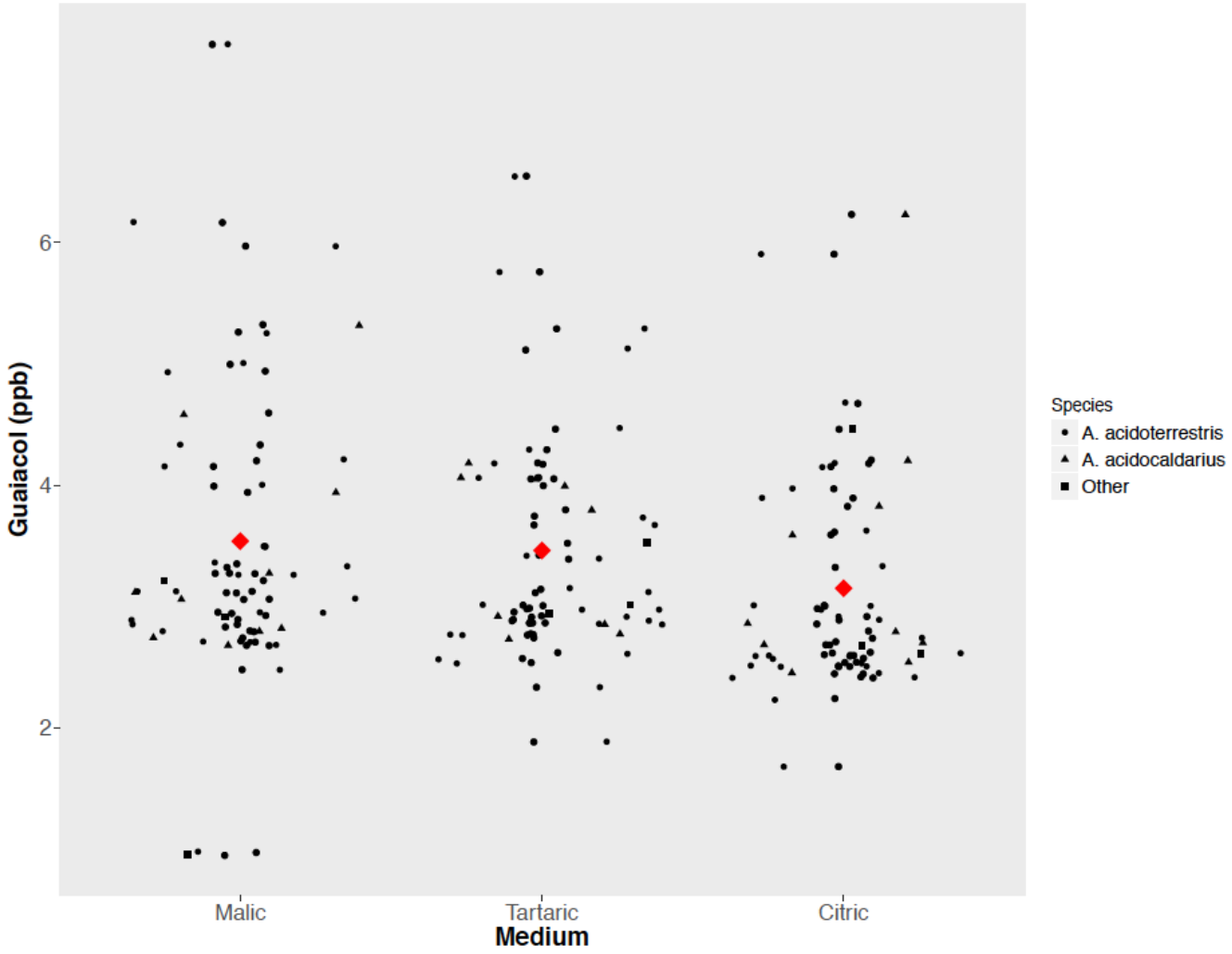

Figure 5.3: Guaiacol concentration after 168 h at 30°C in PDB acidified to pH 3.5 with either malic, tartaric, or citric acid. The reported concentrations are an average of three replicates and were determined using the peroxidase colorimetric assay and recorded at 410 nm. Red diamonds represent the average guaiacol concentrations among all the strains grown in each medium.

Final guaiacol concentrations ranged from virtually non-existent (<1 ppb) to 7.86 ppb. The range of values may become increasingly large were additional vanillin is provided to efficient synthesizers. Many of the guaiacol concentrations localized in the range of 2-3 ppb, which approximates the aromatic detection limit for guaiacol in juices. Isolates which produced >4 ppb guaiacol may be increasingly likely to cause quality deviations in juice products. Moreover, while guaiacol has been adopted as a representative marker for spoilage, other halophenols are recognized by consumers at lower concentrations, which should be considered in assigning spoilage potential (Siegmund and Pollinger-Zierler, 2006). Rapid proliferation (early exponential phase, and high final cell concentration) was determined to be a significant predictor of guaiacol synthesis ($p = 0.017$). Bahceci et al. (2005) concluded that a minimum cell density was required for guaiacol synthesis, but Perez-Cacho et al. (2011) found the association with cell density ambiguous. Similarly, high final cell density was not exclusively predictive in this study. As shown in Fig. 3, isolates produced an extensive range of guaiacol under all three growth conditions. However, citric acid was negatively associated with guaiacol ($p = 0.027$). Isolates are color coded in Fig. 4 according to the growth medium in which they produced the highest level of guaiacol. The acidulants malic and tartaric acid do not appear to be associated with a specific clade, but the paucity of strains for which citric acid induced the greatest guaiacol production is apparent. This strain variability among different media may be a relevant consideration in the development of rapid techniques to differentiate guaiacol from non-guaiacol producers. Lin et al. (2005) developed an FTIR based method for discrimination of the functional category of guaiacol production. Although the method accurately detected

guaiacol producers 89% of time, there was a much higher rate of false negatives (38%). This may be due to the process used to assign function in the training set used to develop the model, if guaiacol production was only assessed initially using a single growth condition. Moreover, the utility of different standardized media in the isolation of Alicyclobacilli from fruit juices may also be dependent on the interspecific variations among isolates. For example, orange serum agar and PDA, typically acidified with tartaric acid, and K agar, acidified with malic acid, each rely on different acidulants.

Whole genome sequencing was performed to identify clade-specific associations with spoilage potential. The majority of the isolates were identified as either *A. acidoterrestris* or *A. acidocaldarius,* although a single *A. acidiphilus* and four *Alicyclobacillus* spp were also identified. The unidentified *Alicyclobacilli* did not cluster with any of the other recognized species for which whole genome data was available. However, these four isolates which were all taken from citrus products, two from New York and two from Florida, were closely related to each other. A maximum likelihood phylogenetic tree was generated from 1,715 SNPs identified across the core genes among these different species (Fig. 4).

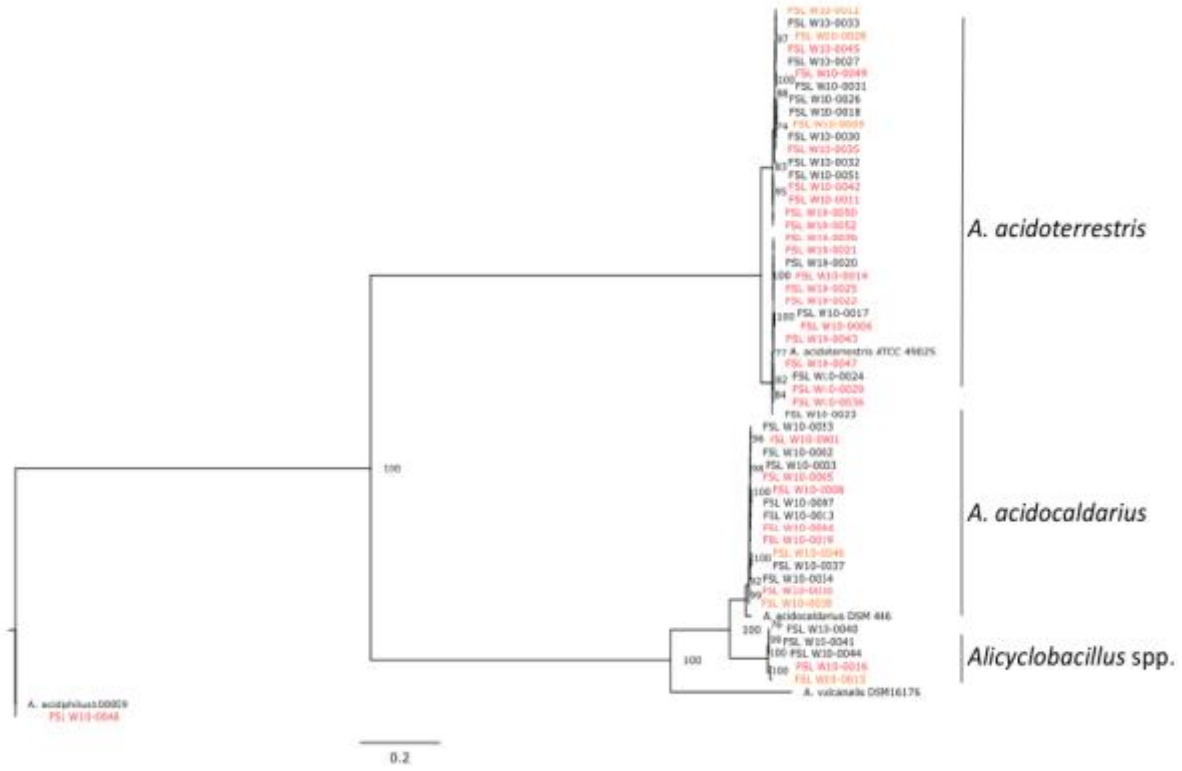

Figure 5.4: Maximum likelihood phylogeny based on single nucleotide polymorphisms in core genes. Bar represents 0.2 substitutions per site. Only bootstrap values >70 are displayed. Isolate names are colored according to the growth medium in which they synthesized the most guaiacol, malic (red), citric (orange), or other (black). Type strains for *A. acidiphilus, A. vulcanis, A. acidocaldarius,* and *A. acidoterrestris* are included for reference.

Six species have previously been shown to produce guaiacol. They are *A. acidiphilus, A. herbarius*, *A. hesperidum, A. cycloheptanicus, A. acidocaldarius,* and *A. acidoterrestris* (Witthuhn et al., 2012). While *A. acidoterrestris* is considered the predominant spoilage-associated species, species was not considered a significant predictor of guaiacol concentration under the conditions utilized in this study. This is due in part to the high level of interspecific and isolate-specific variability observed. The interclass correlation coefficient, the proportion of the total variance in guaiacol production explained by the effect of isolate, was calculated to be 36% (95% CI: 16%-

55%). Additionally, isolate source, although previously determined to be associated with species, was also not a significant predictor of guaiacol synthesis.

5.4 Conclusions

Alicyclobacillus spoilage is associated with the production of off-aromas typified by the medicinal or smoky qualities of guaiacol. Guaiacol synthesis was identified at varying levels across a range of food-sourced *Alicyclobacillus* isolates grown in PDB acidified with differing organic acids and grown at 30°C. The source of the isolate, although associated with species (Durak et al., 2010), was not predictive of guaiacol synthesis (Fig. 3) and did not impact proliferative ability (Fig. 2). However, organic acid-specific effects were observed. Isolates grew preferentially in media with specific but varying acidulants (Fig. 1), and citric acid reduced the level of guaiacol produced compared to isolates grown in medium at the same pH with alternative acidulants (malic and tartaric). Organic acid preferences were not associated with phylogenetic relationships (Fig. 4), suggesting that the genetic determinants associated with juice spoilage are not clade-specific. These findings may contribute to the selection of appropriate target strains in validation and challenge studies, as well as refining the quality management decision making process for juice manufacturers.

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SUPPLIMENTAL FIGURES

Table S1. *Method for classification of strains based on proliferative ability in various media incubated at 45℃.* Strains classified as having high proliferative ability achieved an optical density above 0.6 by the end of 72 h incubation and achieved their greatest increase in proliferation rate (inflection point) within 8 h of incubation. Strains classified as having a medium proliferative ability also achieved a 0.6 OD by the end of 72 h, but the inflection point occurred after 8 h. Low proliferative strains had a final OD less than 0.6.

Category	**Inflection**	**Maximum OD**
Low Proliferative Ability	N/A	<0.6
Medium Proliferative Ability	>8	>0.6
High Proliferative Ability	≤8	>0.6

Table S2. *Method for classification of strains based on proliferative ability in various media incubated at 30℃.* Strains classified as having high proliferative ability achieved an optical density above 0.6 by the end of 168 h incubation and achieved their greatest increase in proliferation rate (inflection point) within 72 h of incubation. Strains classified as having a medium proliferative ability also achieved a 0.6 or greater OD by the end of 72 h, but the inflection point occurred after 72 h. Low proliferative strains had a final OD less than 0.6.

Category	**Inflection**	**Maximum OD**
Low Proliferative Ability	N/A	<0.6
Medium Proliferative Ability	>72	>0.6
High Proliferative Ability	≤72	>0.6